CHARACTERIZATION OF A PUTATIVE ACID PHOSPHATASE IN *TOXOPLASMA GONDII* AND ITS ROLE IN PARASITE PROPAGATION

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The parasite *Toxoplasma gondii* infects approximately one-third of people worldwide. Infection can lead to severe disease in those with a compromised immune system and primary infection during pregnancy can lead to severe birth defects or miscarriage. Treatment options are limited, have significant side effects, and are ineffective for all infection stages. Imperative to the discovery of novel therapeutic targets is a thorough understanding of how *Toxoplasma* propagates within a host. To replicate, the parasite must enter the cells of an infected organism where, during the invasion process, it surrounds itself with host cell membrane to form a parasitophorous vacuole (PV), within which it freely divides. To endure the intracellular environment of a host cell, *Toxoplasma* secretes a large repertoire of proteins beyond the PV to manipulate important host cellular functions. How these *Toxoplasma* proteins transit from parasites to host cell is not well understood. Protein translocation into the host cell is mediated by three proteins hypothesized to function as a putative translocon complex inside the PV, but whether other proteins are involved in the structure or regulation of this putative translocon remains unknown. The secreted protein GRA44, which contains a putative acid phosphatase domain, has been discovered to interact with members of this translocon and is required for downstream alteration of host cells. GRA44 was found to be post-translationally cleaved in a region homologous to sequences targeted by protozoan proteases

of the secretory pathway with both major cleavage products secreted to the PV. Conditional knockdown of GRA44 resulted in loss of host cell cMyc upregulation, a phenotype also seen in translocon member disruption. Therefore, the putative acid phosphatase GRA44, in association with the translocon complex, is critical for host cell manipulation during infection, a process *Toxoplasma* relies upon for successful propagation as an intracellular pathogen.

Gustavo Arrizabalaga, Ph.D., Chair

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List of abbreviations

AIDS	Acquired immune deficiency syndrome
ASP5	Aspartyl protease V
ATC	Anhydrotetracycline
BME	Beta-mercaptoethanol
BSA	Bovine serum albumin
CBD	Chitin binding domain
CDNA	Complementary deoxyribonucleic acid
CMR	Chloramphenicol resistance
CMYC	Cellular myelocytomatosis gene
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DAPI	4',6-Diamidino-2-Phenylindole
DHFR	Dihydrofolatereductase
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERbB-2	Erythroblastic oncogene B-2
FBS	Fetal bovine serum
GAP	Glideosome associated protein
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
Fe	Iron
GRA	Dense granule protein
HA	Hemagluttinin
HFF	Human foreskin fibroblast
HIV	Human immunodeficiency virus
HPT	hypoxanthinexanthine-guanine phosphoribosyltransferase
IFA	Immunofluorescence assay
IMC	Inner membrane complex
IMC2A	Inner membrane complex protein 2A
IP	Immunoprecipitation
IPTG	Isopropyl β-d-1-thiogalactopyranoside
IRG	Iummune-related GTPase
LB	Luria broth medium
MAF1	Mitochondrion association factor 1
Mg	Magnesium
MIC	Microneme protein
Mn	Manganese
MPA	Mycophenolic acid
MRI	Magnetic resonance imaging
MS	Mass spectrometry

MYC	Myelocytomatosis peptide
MYR	Myc regulation protein
OD	Optical density
p53	Tumor suppressor protein p53
pSAG1	Surface antigen 1 promoter
pTUB	Tubulin promoter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEXEL	Plasmodium expert element
PLV	Plant-like vacuole
PNPP	Para-nitrophenol phosphate
PP	Protein phosphatase
PP2C-hn	Protein phosphatase 2C-host nucleus
PPM	Metal-dependent protein phosphatase
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RNA	Ribonucleic acid
RIPA	Radioimmunoprecipitation assay buffer
RON	Rhoptry neck protein
ROP	Rhoptry protein
SAINT	Significance analysis of interactome
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium docecyl sulfate polyacrylamide gel electrophoresis
sgRNA	Small guide RNA
snRNP	Small nuclear riobonucleoprotein
STAT	Signal transducer and activator of transcription
TATI	Transactivator
TEXEL	Toxoplasma export element
TRE	Tetracycline response element
TRIS	Tris(hydroxymethyl)aminomethane
TVN	Tubulovesicular network
UPRT	Uracil ribosyltransferase
UTR	Untranslated region
VAC	Vacuolar compartment
WNG	With-no-gly-loop kinase

Chapter 1: Introduction

Background

Toxoplasma gondii was first discovered in1908 at the Pasteur Institute in Tunis by Nicolle and Manceaux, who were studying leishmaniasis in a small rodent species[1]. Upon realization they had discovered a new organism, Nicolle and Manceaux named it *Toxoplasma gondii,* referencing the Greek word toxos, for bow or crescent based on the shape of the parasite, and *gondii* for the rodent they purportedly studied, *Ctenodactylus gundi*. *Toxoplasma* was also discovered by Splendore the same year in Brazil, but it was not assigned a name, due to initial misidentification as *Leishmania*.

Toxoplasma is a member of the phylum Apicomplexa, which includes other unicellular parasitic species like *Plasmodium spp.*, *Cryptosporidium spp.*, *Cyclospora spp.* and *Babesia spp.*, all of which contain particular apically localized organelles distinct from other eukaryotes. These organisms exhibit complex life cycles with many stages of development and infect a wide variety of hosts and vectors worldwide[2]. In general, *Toxoplasma* exists in two distinct replicative stages: sexual and asexual. Sexual reproductive stages are restricted to felines, making them definitive hosts, while asexual replication can occur in all warm-blooded animals. Animals that can only harbor the asexual stages are considered intermediate hosts.

The life stages of sexual reproduction only occur in the feline gut microenvironment. In the feline gut, parasites invade endothelial cells and form sporozoytes, distinctly large multinucleated cells, before generating gametocytes

for sexual reproduction. Fertilization of a macrogamete by a microgamete results in oocyst formation. Oocysts travel through the feline gut and are shed to the outer environment where they remain viable and infectious to other felines and intermediate hosts.

The life stages of asexual parasites occur in both definitive and intermediate hosts. Asexual parasites are termed either tachyzoites, tachy from the Greek word for "fast", or bradyzoites, from the Greek for "slow". Tachyzoites are a hyper replicative stage directly responsible for host cell and tissue damage resulting from parasite replication[3]. Tachyzoites are thus responsible for acute toxoplasmosis but they are typically susceptible to the immune response. In immunocompetent hosts, tachyzoites convert to the encysted bradyzoite stage as a means to evade the immune response. As bradyzoites, parasites form protective tissue cysts within infected cells and avoid clearance. Accordingly, bradyzoites can remain for the entirety of a host's lifespan establishing a chronic infection. *Toxoplasma* is capable of infecting most tissues, but some tissues appear to be more commonly infected. Specifically, tissue such as muscle/heart and nerve/brain are disproportionately infected and often harbor bradyzoite cysts. These latent tissue cysts are capable of reactivation upon reduced immune pressure, with bradyzoites becoming tachyzoites to resume rapid replication.

Both oocysts and bradyzoite tissue cysts can be infective and consumption of either results in parasites being released by digestive enzymes and invading proximal tissues[4]. Parasite oocysts shed in cat feces can be found environmentally in contaminated water, dirt, sand, vegetation or unwashed

vegetables and direct ingestion of these cysts can result in transmission to other hosts including humans. Similarly, ingestion of tissue cysts in undercooked or raw meat from infected animals can transmit parasites to a new host. Infection from undercooked, cured, or raw meat is most common from wild game or free-range livestock, since these animals have increased contact with the environment and feline species[4,5]. Swine such as commercial hogs or feral pigs have been found to have the highest rates of *Toxoplasma* infection, with poultry such as chickens to be second in seropositivity rates. In summary, *Toxoplasma* has been found capable of infecting almost any nucleated cell of all warm-blooded organisms including mammals and birds and has various modes of transmission including food borne. A wide variety of potential hosts, coupled with an aptitude for long term viable dormancy, has allowed for a breadth of infection not seen in most pathogens.

Health impacts of toxoplasmosis

In humans, *Toxoplasma* is limited to asexual parasite stages and infections are classified as either acute or chronic. An acute infection is characterized by the highly-proliferative tachyzoite parasite form, which is responsible for host cell lysis and ultimately tissue damage and lesions of the infected area if left unchecked by the immune system[6]. Conversely, a chronic infection is characterized by predominance of the bradyzoite parasite form, which undergoes limited replication and produces latent parasite cysts throughout host tissues. Bradyzoites remain mostly dormant, but persist for the duration of the host's life and are capable of transformation back to tachyzoites[7].

Globally, *Toxoplasma* infection is estimated to occur in one-third of the human population, with certain regions of Europe, South America and Southeast Asia having highest infection rates ≥ 60%. In the United States, an estimated 15% of the population is positive for infection[4,8]. Infections typically begin as acute, but are quickly suppressed by host immune pressures, resulting in a mostly asymptomatic chronic infection. Chronic infections can re-activate, however, and become acute upon weakened host immunity. Left unchecked, an acute infection results in severe tissue damage. Toxoplasmic encephalitis and other pathological complications are a significant risk to immunocompromised or immunosuppressed individuals such as organ transplant recipients, HIV/AIDS patients and lymphoma patients[7,9,10]. Toxoplasmic encephalitis has been observed in 30-50% of HIV positive patients with up to 80% of AIDS patients having evident brain lesions as detected by MRI. Of those affected by Toxoplasmosis who are HIV negative, approximately 40% are patients undergoing treatment or in remission from lymphomas. Further, toxoplasmosis can develop congenitally from primary infections in pregnant women, where parasites are passed from the mother to the developing fetus, causing severe birth defects or miscarriage[11]. Although less than 1% of pregnancies are affected by *Toxoplasma* infection, 85% of congenitally infected infants with presumed normal physiology yielded signs of mental retardation and vision loss later in development. While rare, postnatal ocular toxoplasmosis is an infection which can occur in the retina, putting patients at risk of blindness[12].

Upon infecting a host, parasites typically invade nearby cells and begin to replicate. Infection can spread within the infected organism by parasite invasion of host macrophages, which travel to distant tissues and release tachyzoites in new areas. Increasing numbers of free and intracellular tachyzoites can elicit a host immune response. Immune system activation results in typical feverish symptoms mirroring a common influenza infection and is often similar in duration. A healthy immune response can thwart tachyzoite replication and push the parasites into the bradyzoite stage. As bradyzoites, *Toxoplasma* forms protective cysts inside host cells and remains undetectable by the immune system. While encysted parasites have been thought to be mostly dormant, new evidence suggests that they are physiologically active, can divide, and elicit an inflammatory reaction. Whether the encysted stage is associated with disease, including those with inflammatory etiology, has become a focus of significant investigation.

Currently, the recommended treatment for a *Toxoplasma* infection is a combination therapy of pyrimethamine and sulfadiazine[13]. Pyrimethamine counteracts parasite replication by preferentially inhibiting parasite dihydrofolate reductase, a common enzyme important for nucleic acid synthesis, and sulfadiazine disrupts folic acid synthesis by inhibiting the enzyme dihydropteroate synthetase. *Toxoplasma* is unable to obtain folate from its host and therefore must synthesize it *de novo*, making the parasites susceptible to folate synthesis inhibition. This regimen has undesirable side effects, however, and is only efficacious in treating acute toxoplasmosis[14]. Atovaquone has been used as an

alternative treatment which targets the parasites' mitochondria; however increasing drug resistance has reduced its usefulness[15] and there are no approved treatments for a chronic Toxoplasma infection.

Toxoplasma gondii cell biology

Toxoplasma is of eukaryotic origin and exists as a crescent-shaped cell during asexual stages[16]. The parasite has no outer cell wall or protective glycan structure, but just a singular plasma membrane bilayer. Underneath the plasma membrane is a double-membrane complex originating from a series of large flattened vesicles stitched together and termed the inner membrane complex (IMC). Supporting the membrane structures is a complex cytoskeleton largely comprised of microtubules (Figure 1). The apical end of the cell is supported by a conoid complex which contains a set of two apical rings derived from membraneous structure connected atop a large cone-shaped polar ring consisting of 6-8 microtubules wound in a corkscrew configuration. Another ring connects the bottom of this polar ring and supports a set of longitudinal microtubules. There are 22 longitudinal microtubules in total, arranged in a spiral pattern, which cover the majority of the cell. In addition, two internal parallel microtubules originating from the center of the conoid complex span the cell's length.

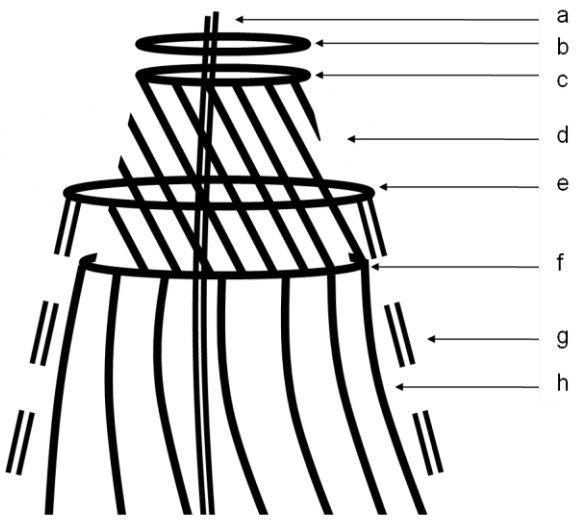


Figure 1. Apical complex of Toxoplasma
Two internal microtubules span the entire length of the parasite(a). The conoid complex consists of apical ring 1 (b), apical ring 2 (c), conoid (d), polar ring 1 (e) and polar ring 2 (f). IMC (g) starts at polar ring 1. Longitudinal microtubules (h) provide outer mechanical support for most of cell length.

When intracellular, parasites reside within a single shared protective vacuole structure, known as the parasitophorous vacuole (PV), which grows in size as the parasites replicate. The PV consists of a single outer membrane, the PV membrane (PVM), and an inner lumen space. The PVM is a nonfusogenic lipid bilayer derived from host cell membrane and contains many embedded parasite proteins[17]. Within the PV lumen are individual parasites and a complex network of actin filaments and vesicles, termed the tubulovesicular network (TVN). In order to obtain necessary nutrients from the host cell, parasites secrete a variety of factors to the PV. The PV contains passive pore structures that allow passage of organic molecules and small peptides up to a size of 1.3 to 1.9 kDa[18,19]. PV membrane transporters and members of the PV pore structures, such as GRA17 and GRA23, are secreted from the parasites to the PVM. The

parasites are vulnerable to host immunity while extracellular due to a lack of innate defense mechanisms and thus tend to quickly invade nearby host cells, constructing a PV on invasion. The PV acts as a barrier to host cell cytoplasm and safeguards against host endolysosomal degradation. If parasites suffer from environmental stressors while in the PV, the PVM is modified with additional proteins and heavy glycosylation to form the cyst wall, shielding parasites from an unsuitable environment.

As a eukaryotic cell, *Toxoplasma* contains common subcellular organelles such as a nucleus, endoplasmic reticulum, Golgi body and a single mitochondrion[16]. Although the parasite has organelles typical of eukaryotes, *Toxoplasma* is auxotrophic for certain nutrients, such as purines, sterols and select amino acids, and must invade a host for continued survival[2]. This nutrient dependency is complemented by a parasitic lifestyle, leading to considerable evolutionary divergence from other phyla[20]. Since *Toxoplasma* relies on a host for survival, they have several parasite-specific organelles to support their biological niche. The micronemes, rhoptries and dense granules are dedicated organelles of the secretory pathway that coordinate induction of necessary parasite secretions to affect the host and expedite parasite propagation. Micronemes and rhoptries are located at the apical end of the parasite, while dense granules are distributed throughout the cell. The apicoplast is another specialized organelle of obscure function. This organelle contains its own DNA and is likely derived from an ancient algae cell, which is presumed to have been engulfed by a *Toxoplasma* ancestral organism[20]. This event is likely to have

established an initial symbiotic relationship and there are relatives of *Toxoplasma* that exhibit photosynthetic abilities supported by an apicoplast. It is evident, however, that *Toxoplasma* itself has lost this photosynthetic trait as it became increasingly adapted to a parasitic life cycle. Instead, the apicoplast of *Toxoplasma* is responsible for *de novo* synthesis of various lipids such as isoprenoids, short-chain fatty acids and heme precursors, thereby remaining an essential organelle. Interestingly, significant exchange of genetic material has likely occurred between the apicoplast and parasite nucleus, giving rise to a mixed genome of both plant-like and mammalian genes that is collectively shared across the two organelles. Lastly, *Toxoplasma* has a small vacuolar structure called the plant-like vacuole (PLV), which associates with another substructure termed the vacuolar compartment (VAC)[21,22]. These structures are not well studied, but it has been hypothesized they are part of an endo-lysosomal system and may coordinate to play a role in ion homeostasis or protein processing due to presence of specifically localized ion channels and to a lower pH within the PLC analogous to endosomes.

Asexual replication gives rise to a cyclical propagation pattern. The first step is extracellular attachment of the parasite to a host cell plasma membrane, followed by indentation of the host membrane at the attachment site and formation of a structure termed the moving junction[6,23]. The moving junction facilitates subsequent invasion of the parasite into the host intracellular space. As a parasite invades, it pulls the host plasma membrane with it to become completely engulfed by a membrane envelope. After invasion, the parasite

pinches off the membrane at the moving junction to form the PV, expelling any remnant host proteins. Once in the PV, parasites replicate asexually through endodyogeny. Endodyogeny is a specific mechanism of cellular division, where two daughter cells are formed within a mother cell and the mother organelles are split between the two forming daughters. As parasites replicate, the PV grows, until a critical point at which the host cell is unsustainable. At this point, the parasites quickly release high levels of intracellular calcium, triggering a controlled egress cascade of the host cell where the PV is deconstructed, host cell membrane is permeabilized and parasites become motile, leaving the host cell remnants behind. Shortly after egress, parasites attach and reinvade neighboring host cells to repeat the cycle. This cycle of attachment, invasion, PV formation, replication and egress can repeat indefinitely, barring interruptive host or environmental factors and has been termed the parasite lytic cycle. Unchecked progression of the lytic cycle results in host cell lysis and tissue damage on a macroscale.

Interactions with the host cell

During the lytic cycle, parasites secrete a wide range of factors to accomplish the steps driving the cycle's progression (Figure 2)[23]. Extracellular parasites secrete attachment factors from the micronemes, which are important for driving motility and attachment to host cells. Upon attachment, the rhoptries begin to secrete factors as micronemal secretions lessen. Proteins secreted from a subcompartment of the rhoptries, known as the rhoptry neck, are first secreted from these organelles along with some late micronemal proteins, that together

establish the moving junction structure. During and after invasion, proteins from the rhoptry bulbs are secreted. Formation of the PV is accompanied by diminished rhoptry secretion and the start of secretion from the dense granules. Dense granule proteins are continuously secreted to set up and maintain structures within the PV, such as the intravacuolar network.

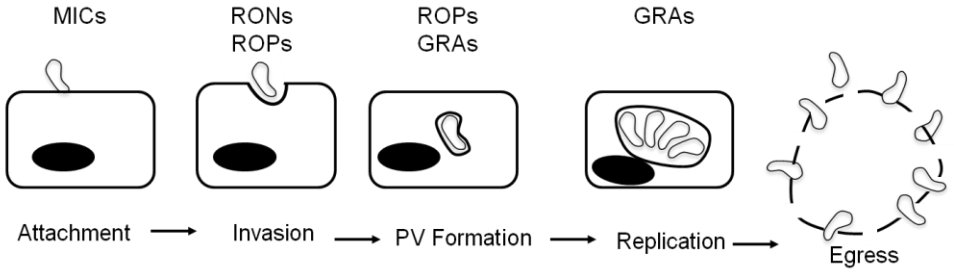


Figure 2. Lytic cycle and protein secretion.
Attachment is mediated by secretion of micronemal proteins (MICs). Rhoptry neck proteins (RONs) are secreted to facilitate invasion, followed by secretion of other rhoptry proteins (ROPs). Dense granule proteins (GRAs) are secreted after establishment of the PV.

To facilitate propagation and survival as an intracellular pathogen, *Toxoplasma* depends upon interaction with the host cell. While in the PV, parasites are safely inaccessible to host degradation pathways, however they are also restricted in their access to host cell structures and systems. To amend this isolated situation, *Toxoplasma* interfaces with host cell processes by secreting an arsenal of protein factors beyond the PV into the host, effectively sabotaging host systems for parasite benefit[2]. Dense granule proteins and some late rhoptry proteins are secreted from the mature PV and play major roles in parasite secretions to the host and host systems manipulation. Many protein factors secreted beyond the PV manipulate host pathways such as transcription, apoptosis, immune response and metabolism. Two well-studied protein kinases

known to be critical for host manipulation are ROP16 and ROP18[24,25]. The kinase ROP16 affects host transcriptional signaling to cause downregulation of STAT3/6 in the host nucleus. This effectively alters host transcription of major gene sets. The kinase ROP18, in coordination with the disordered dense granule protein GRA7, neutralizes innate host cell immune mechanisms by phosphorylation and inactivation of host Immunity-Related GTPase (IRG) proteins[26]. The pseudokinase ROP5 has also been implicated as a cofactor in this neutralization process. Host IRG proteins naturally bind foreign structures, such as the parasite-derived PV, and signal for trafficking to the host endosomal degradation pathway. Recently, the parasite kinases WNG1 and WNG2, have been shown to be important for maintenance of parasite PV membrane integrity[27,28]. Additionally, the dense granule protein GRA16 has shown to be an activator of host cMyc and disregulates p53 upon parasite infection[29]. Amplified cMyc expression can cause increased host cell proliferation and metabolism and altered p53 pathways affect the host's cell cycle.

The mechanisms by which parasite proteins are secreted out into the PV lumen and beyond to the host cytosol and nucleus are largely unknown. To begin understanding this phenomenon, randomly mutagenized parasite populations were screened for loss of protein secretion to host cells[30]. Parasites with deficiencies in secretion were identified by their failure to induce host nuclear cMyc in the respective infected host cell. Parasites failing to upregulate host cMyc were isolated and characterized by genome sequencing. Three genes were identified to contain loss of function mutations leading to the observed secretion

defect[31]. The three gene products localize to the PV and were named MYR1/2/3 (Myc Regulation1/2/3). Furthermore, these proteins were shown to be directly responsible for translocation of other proteins such as GRA16 and GRA24 beyond the PV to the host and are thus indirectly responsible for manipulation of a wide range of host systems[32]. It can then be surmised that in addition to proteins secreted to the host and PV membrane, some specific proteins are secreted to the PV lumen for the purpose of trafficking host-bound factors.

The secretion pathway is understood to contain a Golgi-localized protease, aspartyl protease V (ASP5)[27,33,34]. This protease cleaves a majority of secreted proteins at a consensus motif RRL. The ASP5 recognition site present exclusively in secreted proteins has been termed TEXEL (Toxoplasma Export Element). Studies of ASP5 have shown that its deletion results in uncleaved secreted proteins, but these proteins are nevertheless still secreted. Therefore, the ASP5 enzyme is evidently not highly essential for effector secretion to host cells, in contrast to the MYR1/2/3 proteins. Knockout of ASP5 results in only a partial subset of the host transcriptomic changes as seen for MYR1 knockouts[32]. Site-specific cleavage of secreted proteins is also seen in the related parasite, *Plasmodium spp*., which has an ER-localized protease Plasmepsin V[35]. Plasmepsin V was documented prior to ASP5 and both proteases share a high degree of identity. The consensus motif of Plasmepsin V in Plasmodium is similar to Toxoplasma and is termed PEXEL (Plasmodium Export Element) that has a comparable conserved sequence of RxLxD/E/Q[36].

Phosphatases

In biology, phosphatases have a broad set of roles that center on their primary function of cleaving phosphate groups from substrates. Many phosphatases act on signaling pathways in opposition to kinases and cells use this binary relationship to balance or regulate cellular processes[37–39]. Phosphatases that act exclusively on other proteins as a means of regulation are denoted PP for protein phosphatase[40]. Phosphatases of this type typically consist of a single catalytic phosphatase domain with many binding sites to multiple binding partner domains. When coupled to the phosphatase domain, partner domains confer substrate specificity and binding ability. This process is analogous in concept to the E2-E3 ligase relationship of the ubiquitination pathway. Protein phosphatases are separated into two types: serine/threonine and tyrosine phosphatases. These two types are only capable of dephosphorylating either phosphoserine/threonine or phosphotyrosine residues in their substrates. They can be further classified by protein structure, where phosphatases utilizing a metal structural center are termed PPM phosphatases[41].

Phosphatases do not only act on proteins, however, and many have roles in metabolism where they facilitate necessary chemical reactions of nutrient consumption or organic molecule alteration for trafficking and storage[42]. A distinct class of phosphatases are the metallophosphatases or acid phosphatases. These enzymes have many biological functions and diverse roles[43]. Unlike other phosphatase families, these enzymes are classified by their unusual catalytic mechanism rather than substrate type. Metallophosphatases get their name from

a shared similar metal-dependent active site. In the active site, two metals are coordinated in such a way as to hydrolyze a stabilized phosphate group from the substrate[44]. Contrary to other metal-binding phosphatases, metallophosphatases do not use the metals for structure, but rather for catalysis. Since they utilize metal atoms, this phosphatase type is frequently pH dependent and those active at lower pH are called purple acid phosphatases due to their iron-derived purple hue when in acidic conditions. Metallophosphatases often coordinate an Fe(III) and a second divalent metal atom. Mn(II), Zn(II) or Fe(II) are common secondary metals. Esterolysis of a phosphate group from a substrate occurs through multiple steps beginning with a precatalytic complex originating from two oxygens of the phosphate and the two metals of the active site[44]. A bridging hydroxyl, natively conjugated to the enzyme active site metals, initiates a nucleophilic attack on the complexed phosphate, expelling the substrate as a leaving group. Following substrate release and subsequent electron rearrangements, the bound phosphate is released by reaction with a water molecule to regenerate the native active site configuration containing a hydroxyl bridge. Acid phosphatases serve a wide variety of biological functions across most organisms and are especially common in plants[42]. In humans, these enzymes have documented roles in the downregulation of prostate cell growth through dephosphorylation of ErbB-2 in prostatic cancer and for facilitating osteoclast bone resorption activity by dephosphorylation of bone matrix proteins, such as osteopontin[45,46]. In plants, acid phosphatases have roles in organic phosphate acquisition and pathogen elimination through reactive oxygen species generation[47–49]. Also, an acid

phosphatase in tobacco cells has been found to regulate enzymatic activity of glycosidases found in the cell wall[50].

In *Toxoplasma*, acid phosphatases and metallophosphatases have not been studied extensively. The proteins GAP45 and GAP50, both of which have regions of homology to acid phosphatase domains, have been studied in greatest detail and are important elements of the glideosome complex, a large multi-protein complex responsible for the parasite's gliding motility[51].

Basis of inquiry

Secreted proteins are suggested to alter host signaling pathways through enzymatic activity, such as kinases and phosphatases, and hence are of great interest as potential drug targets. The uniqueness of the Toxoplasma proteome and importance of secreted effectors to parasite propagation make this class of proteins especially attractive for therapeutic intervention. Historically, secreted kinases have been the major focus of study among secreted proteins with potential for drug inhibition.[52] In contrast, the contribution of secreted phosphatases remains largely unexplored. The secreted phosphatase PP2C-hn is the lone example, trafficking to the host cytoplasm and nucleus, but its function remains unclear[53]. To expand understanding of phosphatases secreted to either the host or PV, 32 genes were identified encoding proteins predicted to have a signal sequence and phosphatase domain. From these efforts, I identified TGGT1_228170 as a potential candidate for further study. Although previously identified in brief as part of the inner membrane complex and named IMC2A by Mann and Beckers, this protein contains specific characteristics of a secreted

protein, namely an N-terminal signal sequence and putative TEXEL sites[54]. Additionally, the Tg228170 gene of *Toxoplasma* has been predicted to be essential to parasite fitness as described by Sidik et al. in their work detailing a gene fitness scoring scheme across the entire *Toxoplasma* genome. In their study, each gene of *Toxoplasma* was functionally deleted and resultant changes to parasite fitness were scored on a scale relative to wild-type parasites. Overall, this collection of information paired with the knowledge that *Toxoplasma* utilizes a great degree of secretion-based host manipulation for a parasite advantage led me to hypothesize that the protein, once designated as IMC2A, is a secreted phosphatase important to host manipulation and by extension, parasite propagation.

Aim 1: Determine role of protein processing on localization and function.

To determine the localization of GRA44, I engineered a parasite strain in which the endogenous gene encodes an HA epitope tag at the 3' end. Western blot analysis of the epitope tagged parasites revealed the presence of at least two forms: full length and a 45 kDa protein, which is consistent with the presence of a TEXEL site midway through the protein sequence. Accordingly, a second parasite strain was established containing an internal MYC epitope in addition to the 3' HA epitope for determining localization of all cleavage products. The double tagged GRA44 showed three protein sizes by western blot corresponding to full-length protein, N-terminal, and C-terminal peptides. Immunofluorescence assays using antibodies against both epitopes showed all protein forms are secreted into the parasitophorous vacuole. Disrupting TEXEL processing by

mutation of the consensus motif had no effects on GRA44 localization or function.

Aim 2: Determine GRA44 importance

In order to understand the role of GRA44, I determined its function within the parasite through genetic disruption. As preliminary experiments suggested GRA44 is essential for parasite survival, a conditional knockdown approach was used for gene disruption. Phenotypic analysis of these knockdown parasites showed GRA44 to be essential for parasite growth and propagation.

Aim 3: Identify GRA44 cellular role.

As a putative acid phosphatase, GRA44 has a large number of potential functions and substrates. Acid phosphatases can have roles in metabolism by dephosphorylation of various metabolic intermediates or cellular trafficking by dephosphorylation of certain vesicle phospholipid components. Signaling is also a cellular process where acid phosphatases play a role by dephosphorylation of proteins relevant to signal cascades. Immunoprecipitation of GRA44 identified top interacting proteins, which included the protein MYR1 of the *Toxoplasma* translocation system. Intracellular *Toxoplasma* parasites typically induce changes to host cell biological programs and systems by translocating parasite-derived effectors across the PV membrane via the translocon. I found that GRA44 was necessary for translocon-mediated host cell alteration; specifically GRA44 knockdown parasites failed to activate host cell cMYC. Similarly, MYR1 knockout parasites also exhibit this phenotypic defect.

Chapter 2: Methods

Cell culture

Parasite cell lines were continuously propagated through host cells by regular passage into human foreskin fibroblasts (HFFs). HFF cells were purchased from ATCC. Parasites and HFFs were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM/liter glutamine, 100 units penicillin/mL, and 100 µg streptomycin/ml. Dialyzed FBS was used during periods of pyrimethamine drug selection. All cell cultures, including parasites and separate HFFs, were grown in a humidified incubator at 37°C with 5% CO2. Parasite lines originated from one of two parental RH strains: a strain lacking the hypoxanthine-guanine phosphoribosyltransferase (HPT) gene, referred to as RHΔ*hpt*, and a strain lacking HPT and Ku80, referred to as RHΔ*ku80*[56–58]. Pyrimethamine and chloramphenicol stocks were prepared in ethanol and stocks of anhydrotetracycline (ATc) were prepared in dimethyl sulfoxide (DMSO).

Parasite genetic manipulation and clonal selection

Parasite populations were transfected with genetic constructs by a nucleofection system (Lonza) in provided P3 primary cell buffer and 1 mL cuvettes. To establish independent clonal lines, transfected parasite populations were subjected to limiting dilution followed by clone isolation. Parasite lines were confirmed by immunofluorescence microscopy and western blotting. Construction of DNA plasmid vectors was accomplished with the InFusion HD Cloning Plus system (Clontech) using ligation-independent cloning(LIC) methods. Parent

vectors were linearized by restriction digestion and recombined with PCR-amplified insert DNA. For mutant variations of TGGT1_228170, exogenous vector constructs were altered by site-directed mutagenesis, then transfected into parasites. TEXEL deletions, MYC epitope tag insertion, and TEXEL2 point mutations were accomplished using a Q5 site-directed mutagenesis kit (New England Biolabs), and TEXEL point mutations were similarly accomplished with a QuikChange site-directed mutagenesis kit (Agilent).

For specific targeting of genetic elements to the *Toxoplasma* genome, a CRISPR/Cas9 based approach was used. To expedite the process, a specialized plasmid vector was adopted containing Cas9, driven by the native *Toxoplasma* promoter to Surface Antigen 1 (pSAG1), a guide RNA (sgRNA) driven by *Toxoplasma* U6 RNA promoter and appropriate drug selection cassettes for selective growth in both parasites and bacteria (pSAG1-Cas9-GFP-pU6-sgUPRT)[59]. The original sgRNA is for the parasite uracil ribosyltransferase (UPRT) locus, but was replaced as needed for other genomic targets by site-directed mutagenesis (Q5, New England Biolabs). Two sgRNAs were used, a guide sequence targeting upstream of the 228170 start codon for TATi promoter insertion and another sequence targeting the parasite Ku80 locus for gene complement construct insertion. Genetic constructs for insertion by CRISPR were amplified by PCR to contain 5' and 3' end homology regions to their respective Cas9 cut site. Following PCR amplification, insert DNA was purified by agarose gel extraction then co-transfected with an aliquot of appropriate Cas9-sgRNA vector.

Epitope tagging strategy

To endogenously tag TGGT1_228170 at the C-terminus, a 3'-region directly upstream of the stop codon was amplified by PCR from RHΔ*ku80* parasite genomic DNA and combined with pLIC-3xHA-DHFR vector previously linearized by PacI just upstream of the 3xHA epitopes[58]. Fifty micrograms of the resulting vector was linearized by XcmI and transfected into RHΔku80 parasites. The transfected population was selected for the presence of the pyrimethamine-resistant dihydrofolate reductase (DHFR) allele of the parent vector[60]. To introduce an exogenous copy of TGGT1_228170 into parasites, a vector was generated to contain a section of the genomic TGGT1_228170 locus beginning from the start codon to the stop codon, including all intron segments and appended C-terminal HA epitope. The section of TGGT1_228170 in the vector was flanked by the *Toxoplasma* tubulin promoter with 5'-untranslated region (UTR) and the tubulin 3' UTR. This was achieved by cloning a PCR amplicon of the TGGT1_228170 genomic DNA into the vector pTNRluc-Tub-HPT at NcoI and PacI restriction sites[61]. Fifty micrograms of the resulting vector, pTub-Gra44-HPT, was linearized with ScaI and transfected into RHΔ*hpt* parasites. The transfected population was selected for HPT by maintained passage in media containing mycophenolic acid (50 µg/ml) and xanthine (50 µg/ml). Independent clones were isolated by limiting dilution.

Conditional knockdown and complementation

To generate the GRA44 conditional knockdown strain, a cassette encoding a drug-selective marker, a transactivator (TATi) protein, and a

Tetracycline response element (TRE) was introduced just upstream of the GRA44 start codon. The cassette was amplified from the vector pT8TATi-Gra44-HX-tetO7S[62]. One microgram of this PCR amplicon was transfected into the RHΔ*ku80* parasites already expressing endogenously HA-tagged GRA44. To drive insertion of the TATi cassette, the PCR amplicon was co-transfected with 2 µg of Cas9 vector expressing a sgRNA targeting just upstream of the TGGT1_228170 start codon. Transfected parasites were selected for resistance to mycophenolic acid imparted by HPT, and independent clones established by limiting dilution. TATi cassette insertion was confirmed at the correct locus by PCR. The resulting strain was designatedTATi-GRA44(HA). To obtain a complementedTATi-GRA44(HA) line, a wild-type copy of TGGT1_228170 including a C-terminal MYC epitope tag, driven by a Toxoplasma tubulin promoter, was targeted to the inactive ku80 locus of the TATi-GRA44(HA) strain by CRISPR/Cas9. The insertional cassette, which contains a tubulin-driven copy of TGGT1_228170 and chloramphenicol resistance gene, was amplified by PCR from plasmid pTub-Gra44-myc-CmR with primers to include homology segments to the ku80 locus[63,64]. The pTub-Gra44-myc-CmR vector was constructed by appending a C-terminal MYC tag to the GRA44 gene sequence, which was then amplified from pTub-Gra44-HP and inserted into the pLIC-SMGFP-CmR vector backbone. This engineering strategy effectively replaced the soluble green fluorescent protein (SMGFP) tagging region of the vector with GRA44(MYC), while leaving the associated UTRs and chloramphenicol resistance gene intact.

One microgram of PCR amplicon was co-transfected with 2 µg of the Cas9 vector encoding the Ku80 sgRNA.

Western blot analysis

For qualitative protein detection by western blot, washed parasite samples were resuspended in 2x sample loading buffer with 5% β-mercaptoethanol and boiled for 5 min at 98°C. All boiled samples were frozen at -20°C, then thawed and reboiled for 5 min at 98°C prior to gel loading. SDS-PAGE and western blotting were performed by standard methods, as previously described[65]. For samples of extracellular parasite lysate, parasites underwent natural egress before being collected, then centrifuged, and washed two times with cold phosphate-buffered saline (PBS) (10 min, 1,000xg). For samples of intracellular parasite lysate, infected HFF monolayers, preceding natural egress, were washed 2 times with cold PBS, scraped, and centrifuged for 10 min at 1,000xg.

To quantitatively analyze GRA44 conditional knockdown and complemented lines by western blot, parasites were first grown under normal conditions as described above for 24 hours, then syringe lysed with a 27-gauge needle. Fresh host cells were infected with an equal quantity of syringe-released parasites and grown for 24 or 48 hours in presence or absence of 1 µg/ml ATc. To analyze protein lysates from extracellular parasite samples of these lines, host cells were scraped and parasites released by passage through a syringe followed by centrifugation for 10 min at 1,000 x g. For analysis of intracellular parasite protein lysates, host cell monolayers were washed with cold PBS, scraped, and centrifuged for 10 minutes at 1,000 x g. Resulting samples were

resuspended in 200 µL immunoprecipitation assay lysis solution (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1%Triton X-100) including a protease/phosphatase inhibitor cocktail (Cell Signaling Technology). Lysates were incubated on ice for 1 hour, sonicated 2 times for 15 seconds each that were spaced with 1 minute incubations on ice. Samples were then clarified by centrifugation at 20,000 x g for 15 minutes at 4°C. Supernatants were combined with 4x SDS loading buffer with 10% β-mercaptoethanol (BME) and boiled for 5 min at 98°C. The boiled SDS samples were frozen at -20°C, then thawed and reboiled for 5 minutes at 98°C before gel loading. SDS-PAGE and western blotting were performed as described above. The primary antibodies used for western blotting included rabbit anti-HA at a dilution of 1:1,000 (Cell Signaling Technologies, 3724), rabbit anti-MYC at a dilution of 1:1,000 (Cell Signaling Technologies, 2278), mouse anti-SAG1 at a dilution of 1:2,000 (Genway, GWB51EAB6), and mouse anti-MYR1 antibodies at 1:1,000[30,31]. The secondary antibodies used included peroxidase-conjugated goat anti-mouse and anti-rabbit immunoglobulins at a dilution of 1:10,000.

Immunofluorescence assays

For all immunofluorescence assays (IFA), HFFs were grown to confluence on 1.5-mm glass coverslips before infection with parasites. Infected coverslips were allowed to grow for 20 hours prior to 20 minute fixation in 4% paraformaldehyde. After fixation, cells were washed with PBS, then permeabilized and blocked with a solution of 3% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS for 15 to 20 minutes. Coverslips were incubated with

primary antibodies for 1 hour at room temperature in a solution of 3% BSA and 0.2% Triton X-100 in PBS. These were then washed five times with PBS after primary antibody incubation. Finally, coverslip samples were incubated with fluorophore-conjugated secondary antibodies for 1 hour at room temperature in 3% BSA in PBS, then washed five times with PBS and mounted on glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories). Primary antibodies used were rabbit anti-HA at 1:1,000 (Cell Signaling Technologies, 3724), mouse anti-MYC at 1:1,000 (Cell Signaling Technologies, 2276), rat anti-HA at 1:2,000 (Roche, 11867423001), rabbit anti-human cMyc at 1:1,000 (Abcam, ab32072), mouse anti-gra5 (Biotem, BIO.018.6) at 1:1,000, and mouse anti-gra7 at 1:1,000. The secondary antibodies used (Life Technologies) were Alexa Fluor 488-conjugated goat anti-rabbit (A32731) or goat anti-rat immunoglobulin (A-11006), Alexa Fluor 594-conjugated goat anti-mouse (A-11032) or goat anti-rabbit immunoglobulin (A-11012), and Alexa Fluor 647-conjugated goat anti-mouse immunoglobulin (A-21235). All secondary antibodies were used at dilutions of 1:2,000. Images were taken on a Nikon Eclipse 80i microscope using a Nikon DS-Qi1Mc camera and NIS Elements AR (v3.0) software.

Immunoprecipitation and proteomics

Immunoprecipitation experiments with intracellular samples were accomplished by harvesting infected host cells prior to egress. HFF monolayers were washed 2 times with cold PBS and scraped from the flask surface to collect intracellular parasites, which were centrifuged for 10 minutes at 1,000 x g and

resuspended in 200 µL ice-cold IP lysis buffer (Pierce, Thermo Scientific) containing protease and phosphatase inhibitors (Cell Signaling Technology). Lysates were incubated on ice for 1 hour, sonicated on ice 2 times for 15 seconds each time, then centrifuged for 15 min at 20,000xg and 4°C. Supernatants were collected and incubated with magnetic beads conjugated to either mouse immunoglobulin or primary antibody (Pierce, Thermo Scientific) for 1 hour at 4°C with rocking. After incubation, beads were separated from solution by magnetism and washed with IP lysis buffer (Pierce, Thermo Scientific) containing inhibitors 3 times and either stored in 8 M urea at -80°C for downstream mass spectrometric analysis or directly eluted into 2x SDS sample loading buffer with 5% β-mercaptoethanol, boiled for 5 min at 98°C, and stored at -20°C for Western blot analysis. SDS-PAGE and western blotting were performed as outlined above. Samples for mass spectrometry were denatured and digested with trypsin before analysis by an Orbitrap Fusion Lumos mass spectrometer. Protein mass spectrometry analysis was completed by the Indiana University School of Medicine Proteomics Core facility. Results were processed by the SAINT (Significance Analysis of Interactome) computational program for statistical quantification and to define significant protein interactions[66]. A SAINT probability of 0.8 was used as a cutoff and interactors ranked by the most stringent fold change metric (Fold Change B).

Parasite growth assays

Twelve-well tissue culture plates of confluent HFFs were infected with 500 parasites/well of freshly syringe-lysed parasites, and parasites allowed to grow

undisturbed for 5 days before fixation with methanol for 5 min. Fixed wells were stained with crystal violet, and plaque images quantified with ImageJ software using the Colony Area plug-in[65].

Host c-Myc quantitation assay

Parasites used for c-Myc assays were grown for 48 hours with or without 1 µg/ml ATc and syringe lysed prior to infection of host cell coverslips. Coverslips of confluent HFF monolayers were serum starved by pretreating for 24 hours with FBS-free medium before being infected. Cells were fixed 19 hours post-infection. IFAs for human cMyc, HA, and DAPI were performed as described above. Images of phase-contrast, DAPI, HA, MYC, and cMyc channels were acquired for at least 20 vacuoles under each experimental condition and exported to ImageJ software. Merged-channel images were used to identify infected host cell nuclei and then quantitated for cMyc expression from corresponding images of the cMyc channel alone. Mean pixel intensity was measured within the host nucleus boundaries of singly infected cells containing PVs with greater than one parasite. Experiments were done in triplicate and measurements averaged.

Recombinant expression of GRA44 phosphatase domain

Recombinant protein expression of the GRA44 phosphatase domain was accomplished with the IMPACT expression system utilizing the pTXB1 plasmid (New England Biolabs) in Rosetta bacterial cells (Novagen). A section of the GRA44 gene encoding amino acids 331 to 693 was amplified from *Toxoplasma* cDNA and cloned into pTXB1 plasmid expression vector with an appended N-terminal methionine. Competent bacteria were transformed with the expression

vector by heat shock at 42°C and glycerol stocks made for storage at -80°C. Bacterial starter cultures were grown overnight in LB medium with appropriate antibiotics at 37°C prior to induction. To induce protein expression, an aliquot of fresh cells from a starter culture were grown at 37°C to an OD_{600} of 0.5 before addition of IPTG and 10 µM magnesium sulfate to the media. Induction cultures were incubated 18 hours overnight at 30°C and cell pellets obtained by centrifugation at 11,000 x g at 4°C for 10 minutes. Proteins were separated and visualized by SDS-PAGE and coomassie staining. Recombinant protein solubility was assessed by sonicating samples 3 times for 10 seconds in buffer containing 20mM Tris/HCl, 300mM NaCl, and protease inhibitors (Roche). Lysate was centrifuged 20,000xg for 15 minutes at 4°C and supernatant isolated from the insoluble pellet. Both the soluble supernatant and insoluble pellet fractions were analyzed by SDS-PAGE and coomassie stain.

GRA44 phosphatase activity from parasite lysate

To measure GRA44 phosphatase activity from purified parasite lysates, samples were assessed by ELISA methods. 96-well plates were pretreated with Rabbit anti HA antibodies at a concentration of 1:1000 overnight at 4°C in sodium bicarbonate at pH 9. Plates were washed 5 times with PBS and blocked 1 hour with 5% BSA in PBS before introduction of samples. Parasite samples consisted of extracellular parasites harvested as described above, but with sonication in RIPA buffer containing EDTA-free protease inhibitors (Roche) for 3 times, 10 seconds each on ice. Goat anti rabbit conjugated to alkaline phosphatase was used as a positive control at a dilution of 1:100 and pH of 8. All samples were

incubated 1 hour at room temperature, washed 5 times with PBS, and incubated with para-nitrophenyl phosphate (PNPP) 30 minutes at 37°C. Absorbance was measured at 405 nm with a Biotek Synergy H1 microplate reader.

Chapter 3: Results

Bioinformatic identification of secreted phosphatase candidates

A bioinformatics approach was used for identifying putative secreted phosphatases from annotated genes of ToxoDB, a *Toxoplasma gondii* genome database (toxodb.org). The initial BLAST search of all genes within ToxoDB was filtered by including only genes whose products contain predicted phosphatase domains and signal peptides. This generated a list containing 32 proteins of potential interest (Table 1). In order to identify proteins likely to have important function in parasite propagation, candidate genes were ranked according to gene fitness scores as assigned by a genome-wide CRISPR/Cas9 knockout study[67]. Among the top candidates, TGGT1_228170 contains a predicted acid phosphatase domain and, despite having a signal sequence, was previously described as being localized to the inner membrane complex (IMC). However, further information suggested that the TGGT1_228170 product may indeed be secreted. Firstly, the related apicomplexan parasite *Plasmodium berghei* expresses a homologous protein UIS2, which also has a secreted ortholog (Pf3D7_1464600) in *Plasmodium falciparum*. Secondly, TGGT1_228170 has been frequently detected in BioID experiments of the PV lumen and PV membrane as an interactor of proteins localized to these microenvironments. Lastly, closer analysis of the Tg228170 protein sequence reveals multiple putative *Toxoplasma* export elements (TEXEL), originally defined by the consensus sequence RXLXD/E although recently refined to the consensus RRL. TEXEL sequences are recognized and cleaved by aspartyl protease V (ASP5) in

the Golgi as part of the secretory pathway to the PV/PV membrane (PVM) and host cell[34]. Together, this additional information and the fact that TGGT1_228170 was assigned a gene knockout fitness score of -3.28, indicating an essential function to parasite fitness, I decided to revisit the localization and importance of this protein.

ID number	Product Description	Fitness score
TGGT1_283720	phosphotyrosyl phosphate activator (ptpa) protein	-4.71
TGGT1_224920	hypothetical protein	-4.56
TGGT1_311290	protein tyrosine phosphatase family protein, ptpla protein	-3.86
TGGT1_283590A	NLI interacting factor family phosphatase	-3.76
TGGT1_219320	acid phosphatase GAP50	-3.74
TGGT1_228170	inner membrane complex protein IMC2A	-3.28
TGGT1_276210	phosphoglycerate mutase family protein	-2.94
TGGT1_216600	exonuclease III APE	-2.67
TGGT1_237410	protein phosphatase 2C domain-containing protein	-2.01
TGGT1_204080	histidine acid phosphatase superfamily protein	-1.99
TGGT1_243990	Dullard family phosphatase domain-containing protein	-1.94
TGGT1_224220	serine/threonine-protein phosphatase PP2A catalytic subunit	-1.5
TGGT1_305910	hypothetical protein	-1.31
TGGT1_252380	hypothetical protein	-1.01
TGGT1_259960	Nucleoside-diphosphatase	-0.67
TGGT1_204410	endonuclease/exonuclease/phosphatase family protein	-0.49
TGGT1_201630A	protein phosphatase 2C domain-containing protein	-0.06
TGGT1_277720	GDA1/CD39 (nucleoside phosphatase) family protein	0.28
TGGT1_244450	protein phosphatase 2C domain-containing protein	0.51
TGGT1_228160	acid phosphatase	0.55
TGGT1_278878	GDA1/CD39 (nucleoside phosphatase) family protein	0.6
TGGT1_268770	dual specificity phosphatase, catalytic domain-containing protein	0.71
TGGT1_278882	GDA1/CD39 (nucleoside phosphatase) family protein	0.71
TGGT1_276920	protein phosphatase 2C domain-containing protein	0.75
TGGT1_308950	histidine acid phosphatase superfamily protein	0.77
TGGT1_225290	GDA1/CD39 (nucleoside phosphatase) family protein	0.81
TGGT1_278510	protein phosphatase 2C domain-containing protein	1.01
TGGT1_237500	protein phosphatase 2C domain-containing protein	1.13
TGGT1_270320	protein phosphatase 2C domain-containing protein	1.24
TGGT1_297650	Ser/Thr phosphatase family protein	1.52
TGGT1_222840	Ser/Thr phosphatase family protein	2.25

Table 1. Proteins identified from bioinformatics search.
The *Toxoplasma* genome was searched and filtered for genes encoding proteins likely to be secreted phosphatases of essential cellular function. The search yielded 32 genes in total, which included TGGT1_228170 sixth when ranked by gene knockout fitness score.

Localization of TGGT1_228170

To characterize the functions of TGGT1_228170, I introduced a DNA sequence encoding three C-terminal hemagglutinin (HA) epitope tags (3xHA) upstream and adjacent to the stop codon of the endogenous gene locus by homologous recombination (Figure 3). Protein extract from both intracellular and extracellular parasites of the TGGT1_228170(HA) line showed a band of approximately 180 kDa by western blot, which is the expected size for full-length protein. However, a second prominent band at approximately 40 kDa was also noted in both intracellular and extracellular parasite samples (Figure 3B). This smaller band is consistent with processing at either of two sequences with homology to TEXEL sites. The sequence for the first of these putative cleavage sites is RELEE (amino acids 1205 to1209), consistent with the former TEXEL consensus, while the second sequence is RRLLE (amino acids 1348 to 1352), consistent with the updated RRL consensus (Figure 3A).

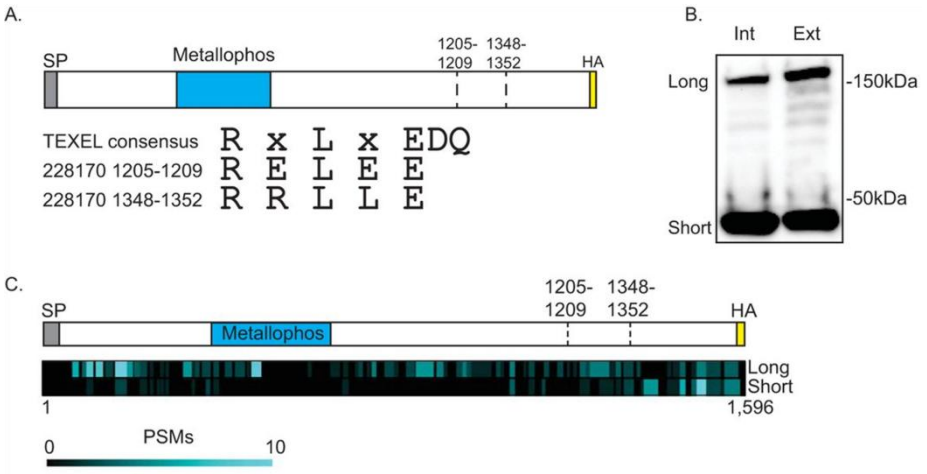


Figure 3. TGGT1_228170 is processed into at least two forms. Schematic for HA epitope tagged 228170 gene (A) showing locations of signal peptide (SP), phosphatase domain, putative TEXEL sequences, and 3x HA epitopes (HA). Lysate preparations from intracellular and extracellular parasites

showed long and short bands by western analysis (B). MS analysis of long and short bands identified the short band as predominantly C-terminal peptides (C).

Samples were further analyzed by mass spectrometry (MS) to confirm the identity of the smaller protein band. To accomplish this, endogenously tagged TGGT1_228170 protein was immunoprecipitated and the eluate separated by SDS-PAGE. Both the 180-kDa (long) and 40-kDa (short) bands were excised from the PAGE gel and analyzed as separate samples (Figure 3C). Results confirmed that both bands corresponded to TGGT1_228170. A total of 192 peptides were detected for the long band with a mapped distribution covering the full protein sequence. MS analysis of the short band revealed a total of 74 peptides corresponding to TGGT1_228170 with 60 of these mapping specifically to regions after the second putative TEXEL sequence (Figure 3C). Therefore, TGGT1_228170 is processed and both the full-length protein and C-terminal fragment are stable.

To determine protein localization, immunofluorescence assays (IFAs) were performed of intracellular TGGT1_228170(HA) parasites (Figure 4). Consistent with the presence of a signal sequence and putative TEXEL sites, TGGT1_228170 was detected within the PV lumen and at the PV membrane (PVM) in particular (Figure 4, arrows). Based on this localization and corroborative findings by Coffey et al[27]. for the same protein, TGGT1_228170 should be designated a GRA protein, and henceforth is referred to as GRA44.

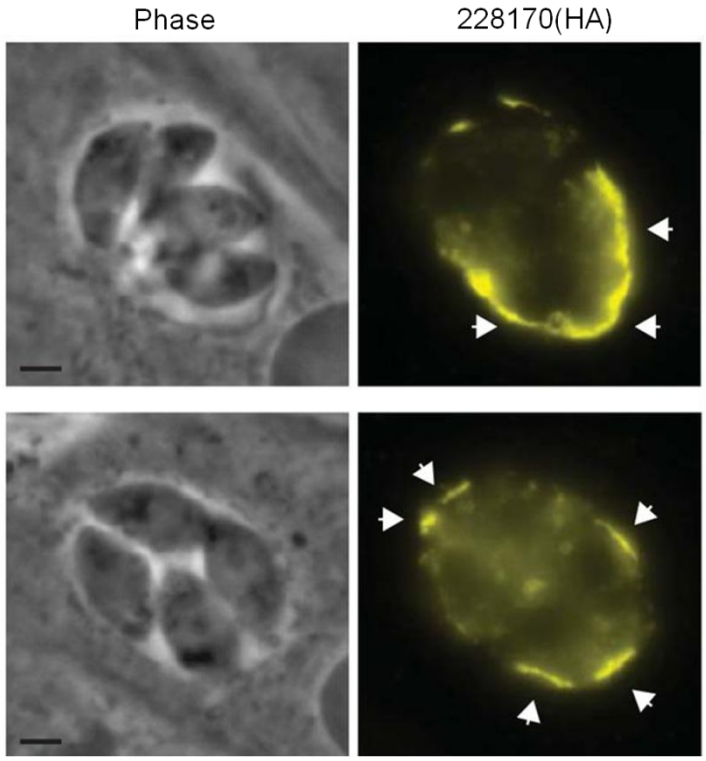


Figure 4. GRA44 is secreted into the parasitohporous vacuole. Immunofluorescence assay (IFA) of intracellular parasites expressing TGGT1_228170(HA) stained for HA in yellow. Endogenously tagged GRA44 localizes to PV lumen and PV membrane. PV membrane localization was observed to be dynamic and at highest intensity (arrows). Scale bars are 2 µm.

TGGT1_228170 is cleaved at TEXEL site

The small C-terminal fragment of GRA44 detected by western blotting and MS is the product of cleavage at either of two putative TEXEL sequences which are located approximately 100 residues apart. To investigate which of the two sites is cleaved if not both, GRA44(HA) mutant variants were exogenously expressed with the first arginine of either TEXEL site or both sites switched to alanine (Figure 5A). Mutating the first arginine of TEXEL sites to alanine has previously been shown to reduce cleavage. Mutation of the first putative site (GRA44 R1205A) did not affect processing of the protein as detected by Western

blot. However mutation of the second site (GRA44 R1348A) significantly reduced processing and short band density (Figure 5B). The same result was observed when both putative TEXEL sites were mutated (GRA44 R1205A/R1348A). Densitometry analysis showed that the wild-type version of the protein was effectively cleaved (82.9%±9.9%, n=3), and processing of GRA44 R1348A was reduced, (47.4%±9.9%, n=3). Thus, it appears that the second TEXEL of GRA44 is processed, and will be referred to as the GRA44 TEXEL. Although a significant reduction in cleavage was observed after altering these single amino acids in the GRA44 TEXEL, there appeared to be some residual C-terminal cleavage product present in all cases. To ascertain whether this was the effect of persistent cleavage, despite the mutations, or cleavage at an alternative site, I generated an exogenously expressed GRA44 mutant in which all 5 amino acids that make up this TEXEL (amino acids 1348 to 1352) were deleted from the protein sequence (GRA44 Δ1348–1352). As expected, TEXEL deletion resulted in a significant loss of the C-terminal cleavage product (Figure 5C). However, it was evident that there still remained a measurable amount being cleaved. The cleavage level for theΔ1348–1352 mutant was calculated to be 21.1%±7.7% (n=3), which was significantly lower than that for the R1348A point mutant.

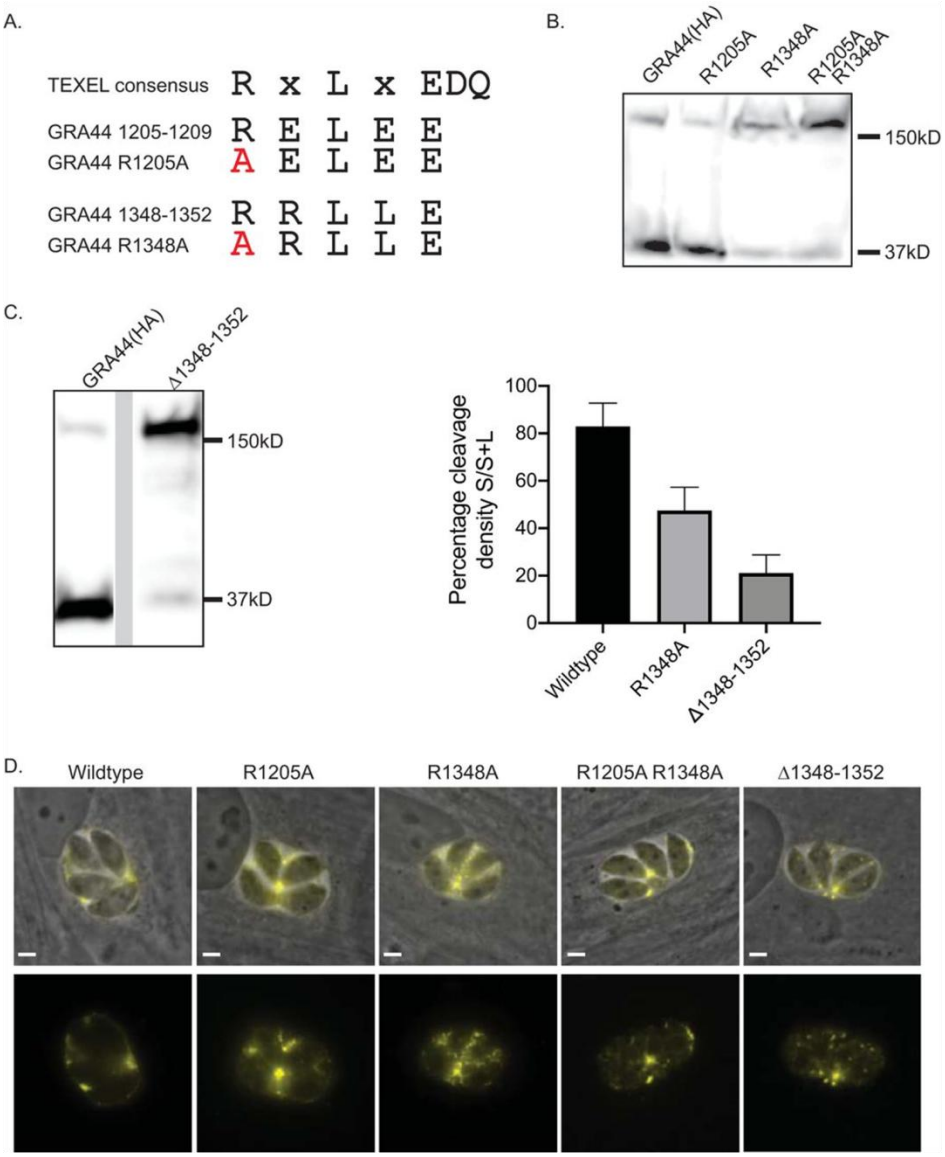


Figure 5. TEXEL 2 of GRA44 is predominantly cleaved.
To determine the site responsible for the processing of GRA44, exogenous copies of mutated GRA44(HA) were expressed in parasites where the first arginine of each putative cleavage site was mutated to alanine individually (R1205A and R1348A) or simultaneously (R1205A/R1348A) An additional mutant of GRA44(HA) was expressed in which the 5 amino acids of the second putative TEXEL site were deleted (Δ1348–1352). (A) Diagram of the TEXEL consensus sequence and putative sites in GRA44, with mutated amino acids indicated in red. (B) Western blot of lysates from parasites expressing the exogenous GRA44(HA), the R1205A or R1348A mutant version, or the double R1205A/R1348A mutant probed for HA. (B) (Left) Western blot of lysates from parasites expressing an exogenous copy of GRA44

lacking the second site (the Δ1348–1352 mutant) probed with antibodies against the HA epitope tag.(Right) Percent cleavage in the wild-type, R1205A, and Δ1348–1352 strains, determined by calculating the ratio of the density of the large band (L) over the sum of the density of both bands (SL).The results for all data sets (n=3, mean ± SD) were significantly different from those for the others, based on one-way analysis of variance ($P<0.05$). (D) Representative images of intracellular parasites expressing each of the four GRA44 processing mutants. Images are of the immunofluorescence signal from the HA tag (in yellow) and of the HA signal overlaid on the phase image. Bars=2 μm.

For a thorough examination of the identified TEXEL, I generated parasites exogenously expressing GRA44 in which either L1350 or E1352 was mutated to alanine. As was the case for the R1348A mutation, changing the central leucine to an alanine disrupted cleavage processing (Figure 6); however, mutant E1352A showed levels of processing similar to wild-type (Figure 6). Since TEXEL cleavage persisted despite all mutations and outright sequence deletion, it is plausible that a cryptic site, potentially amino acids 1205 to 1209, was being cleaved instead. Alternatively, GRA44 may be cleaved by a TEXEL/ASP5-independent mechanism. IFAs were performed of parasite lines expressing each of the four mutant GRA44 proteins (R1205A, R1348A, R1205A/R1348A, and Δ1348–1352 mutants) to determine whether effective processing is needed for correct localization to the PV. Interestingly, none of the mutations affected secretion or localization to the PV (Figure 5D). Similarly, mutating L1350 or E1352 within the confirmed TEXEL site had no affect on PV localization of GRA44 (Figure 6C). Based on these results, complete processing at the TEXEL site is not required for protein secretion.

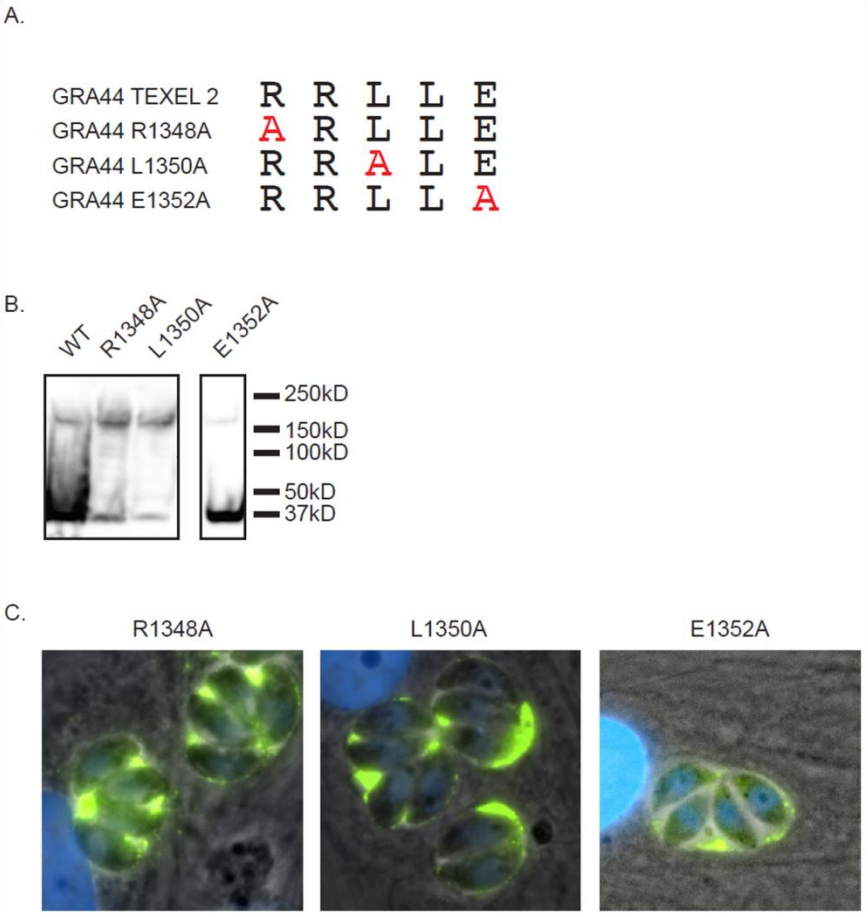


Figure 6. Further TEXEL mutations showed no effect on secretion. Exogenous copies of GRA44(HA) in which each of three conserved residues of the RxLxD/E motif were individually mutated in the primary TEXEL were expressed in parasites to assess effects on cleavage efficiency and secretion. (A) Diagram of primary TEXEL showing WT amino acid sequence and mutated sequences with specific residues mutated to alanine indicated in red. (B) Western blot of parasite lysates showed mutation of leucine to alanine (L1350A) resulted in cleavage reduction, while mutation of glutamate (E1352A) had no observable effects. (C) IFA of intracellular parasites exogenously expressing mutant GRA44(HA) constructs reveal these mutations also had no observable adverse effects to GRA44 secretion.

Cleavage products colocalize in PV

Initially, the GRA44(HA) parasite lines used for study were constructed with C-terminal HA epitope tags for antibody detection. Since GRA44 is cleaved

at an internal TEXEL site, only full-length uncleaved protein and the post-cleavage smaller C-terminal fragment could be detected by western blot and IFA. As a result, localization of the N-terminal cleavage product, which contains the putative acid phosphatase domain, could not be determined with these parasite lines. Accordingly, I engineered a parasite line to exogenously express GRA44 with an internal MYC epitope tag inserted between amino acids 1203 and 1204, along with a C-terminal HA epitope tag (Figure 7A). Protein extracts from parasites expressing the dually tagged GRA44 were analyzed by Western blot and probed separately with antibodies against the MYC or HA epitopes (Figure 7B). Probing of anti-MYC uncovered an additional band at approximately 140 kDa, which correlates to the expected N-terminal end of GRA44 post-cleavage. Probing of anti-HA showed full-length GRA44 and the C-terminal fragment similar to previous experiments. After establishing this parasite line to allow for monitoring of both N-terminal and C-terminal post-processing fragments of GRA44, I investigated their respective localizations by IFA (Figure 7C). Regardless of whether HA or MYC antibodies were used, the protein was localized primarily to the PV, which suggests that the two major polypeptides, including the one containing the phosphatase domain, are secreted into the PV.

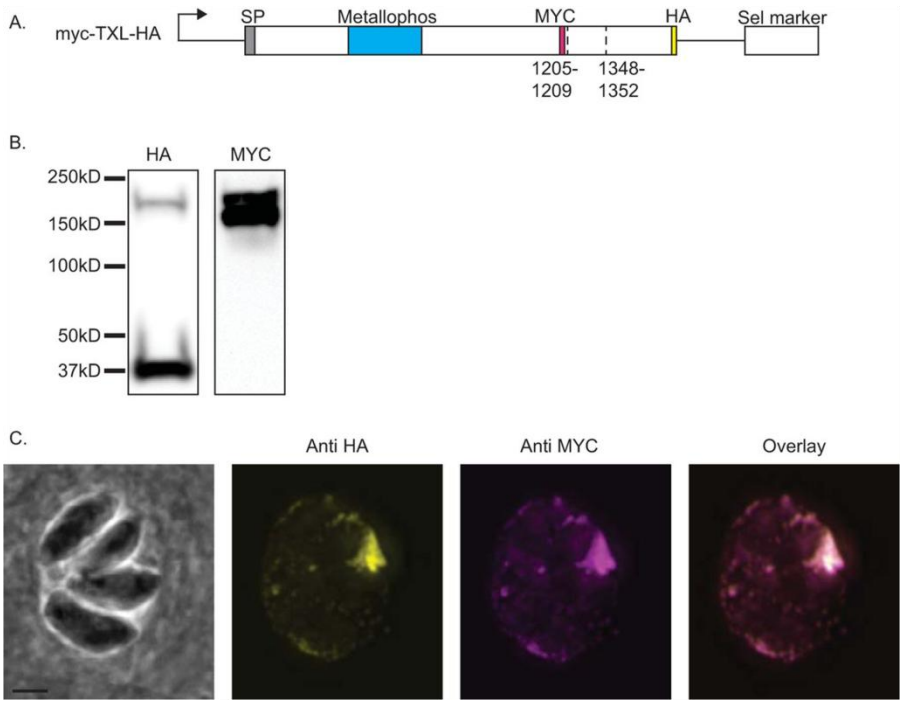


Figure 7. N-terminal cleavage product of GRA44 is secreted.
(A) To determine size and localization of the GRA44 N-terminal cleavage product, an exogenous copy of GRA44 with an internal MYC epitope tag and a C-terminal HA epitope tag was expressed in parasites. (A) Schematic of the exogenous GRA44 construct MYC-TXL-HA illustrating positions of the MYC and HA epitope tags relative to the both putative TEXEL motifs. (B) Western blot of the MYC-TXL-HA parasite line separately probed with anti-HA and anti-MYC antibodies showed a total of three bands for GRA44. (C) IFA images of parasites expressing MYC-TXL-HA probed for HA (yellow) and MYC (magenta). Bar=2 µm.

Knockdown compromises parasite growth

A genome-wide CRISPR screen had previously assigned GRA44 a $\log_2$ relative fitness score of -3.28, which would suggest that loss of the protein would be significantly detrimental to parasite propagation. Due to this predicted importance to fitness and survival, I applied a tetracycline (Tet)-repressible system to establish a conditionally controlled GRA44 knockdown line. More specifically, CRISPR/Cas9 was used to introduce a cassette encoding a

transactivator (TATi) protein and tetracycline response element (TRE) just upstream of the endogenous GRA44 start codon (Figure 8A). The conditional knockdown line was engineered using the endogenously tagged GRA44(HA) parasites for straightforward monitoring of GRA44 protein expression. The resulting parasite line,TATi-GRA44(HA), was grown for 24 or 48 hours in the absence and presence of the tetracycline analog anhydrotetracycline (ATc), and GRA44 expression monitored by Western blotting and IFA. At both time points, protein levels were significantly reduced in the presence of ATc (Figure 8B and C). Thus, the conditional knockdown feature functioned properly in the parasite line. Interestingly, the small C-terminal fragment from the TATi-GRA44(HA) parasites was smaller than what was observed with the parental endogenous HA-tagged strain. Sequencing of GRA44 in the TATi-GRA44(HA) line showed that a 315-bp fragment, encoding the last 105 amino acids, was deleted, but with the HA tag in frame. Surprisingly, no significant propagation defects were detectable and this line was successfully complemented with a full-length gene construct (compare the results of the parental parasites to those of the knockdown grown without ATc). Thus, deletion of the region does not affect localization or function. Regardless, this strain allowed for studying effects of eliminating GRA44 expression with ATc addition. Importantly, when grown in the presence of ATc, depleting GRA44, propagation of TATi-GRA44(HA) parasites was significantly affected as compared to their propagation under normal conditions (Figure 8D). For the conditional knockdown strain grown in absence of ATc, an average cell clearance of 19.4%±9.9% was quantitated, which was

reduced to 0.9%±0.4% on addition of ATc to the growth medium. So, conditional knockdown of GRA44 significantly reduces parasite propagation in tissue culture.

To confirm that the propagation defect was due to reduction of GRA44 levels, TATi-GRA44(HA) parasites containing an exogenous copy of GRA44 were developed to complement the phenotype. A copy of GRA44 with a C-terminal MYC epitope tag driven under tubulin was introduced to the TATi-GRA44(HA) line at the endogenous Ku80 locus to generate the complement line, TATi-GRA44(HA)comp, (Figure 9A). The exogenous copy of GRA44(MYC) was processed and secreted as expected (Figure 9B and C). Without ATc, both the endogenous HA-tagged GRA44 and the exogenous MYC-tagged GRA44 were detected in this line by Western blotting and IFA (Figure 9B and C). As expected, addition of ATc resulted in knockdown of GRA44(HA) but had no effect on exogenous GRA44(MYC) expression. Plaque assays of TATi-GRA44(HA) and TATi-GRA44(HA)comp parasites were performed in parallel with and without ATc to determine if the GRA44 extra copy complemented the propagation defect. Consistent with previous results, addition of ATc to TATi-GRA44(HA) severely impaired growth, however TATi-GRA44(HA)comp parasites were relatively unaffected by presence of ATc (Figure 9D). The percent clearance of host cell monolayer after 5 days in culture with ATc was 0.8%±0.4% for the knockdown strain and 26.6%±3.7% for the complemented strain, statistically equal to clearance observed without ATc by either parasite line. Complementation of the plaque formation phenotype by addition of a wild-type copy confirms that GRA44 is critical for the efficient propagation of *Toxoplasma*.

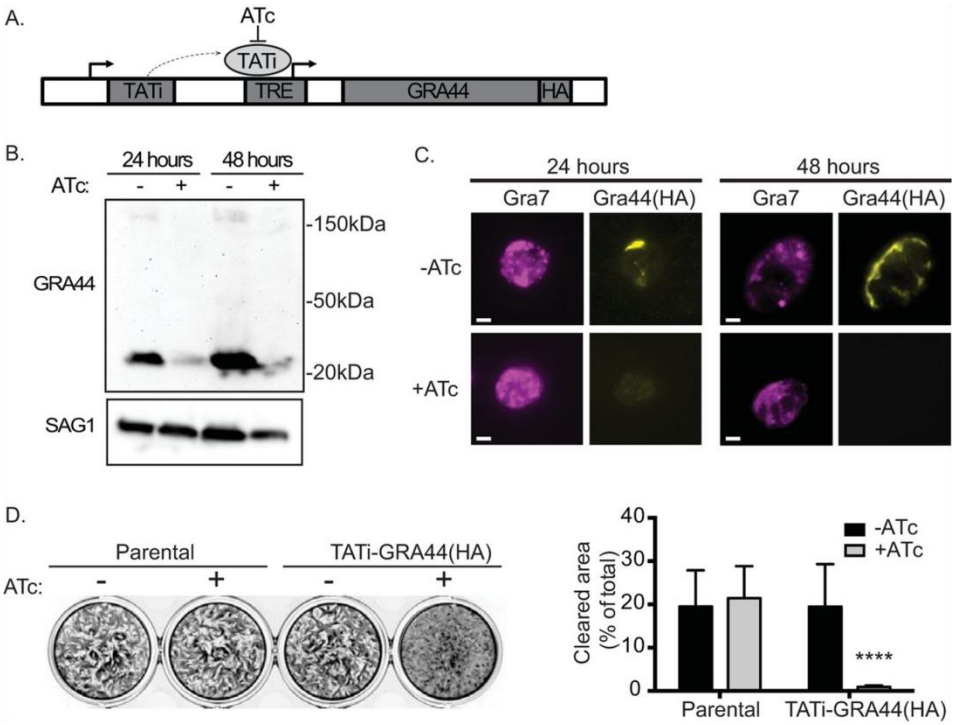


Figure 8. Conditional knockdown of GRA44 stalled parasite growth. To determine the function of GRA44, a tetracycline (Tet)-repressible system was applied to establish a conditional GRA44 knockdown parasite line. (A) Schematic of GRA44 conditional knockdown strategy (B) Quantitative Western blot of TATi-GRA44(HA) parasites grown for either 24 or 48 hours in the absence or presence of ATc. Blots were probed with HA antibodies to detect GRA44 with SAG1 used as a loading control. (C) The reduction in GRA44 expression in the presence of ATc was confirmed by IFA of intracellular TATi-GRA44(HA) parasites grown with and without ATc for 24 or 48 hours and probed with anti-HA antibodies. Bars=2 µm. (D) Plaque assays were performed with GRA44(HA) parasites (parental) or TATi-GRA44(HA) parasites grown with or without ATc for 5 days. (Left) Representative plaque assay images. (Right) Results were quantified based on the percentage of cell monolayer cleared by parasite propagation (cleared area) and the average for biological and experimental triplicates (n=3; mean ± SD; $P<0.0001$, unpaired t test).

Cleavage and function

The GRA44 protein has an essential function in parasite propagation and is cleaved at an inner TEXEL sequence before being secreted into the PV. To

determine if the processing of this TEXEL is needed for function, the TATi-GRA44(HA) strain was complemented with an exogenous copy of GRA44(MYC) containing a deletion of TEXEL residues 1348 to 1352, giving the GRA44compΔTXL parasite line. For GRA44compΔTXL parasites under normal growth conditions, anti-HA probing detected endogenous GRA44(HA) by western blotting, similar to that in the TATi-GRA44 and TATi-GRA44comp lines. Probing blots with anti-MYC antibodies detected the exogenous MYC-tagged GRA44compΔTXL copy as mostly full-length protein, unlike the TATi-GRA44comp, which was processed (Figure 9E). Under knockdown conditions with ATc, the GRA44compΔTXL complemented parasite line remained unaffected (Figure 9F) and exhibited relatively normal host cell clearance. These results indicate that the complete cleavage of GRA44 is not necessary for function.

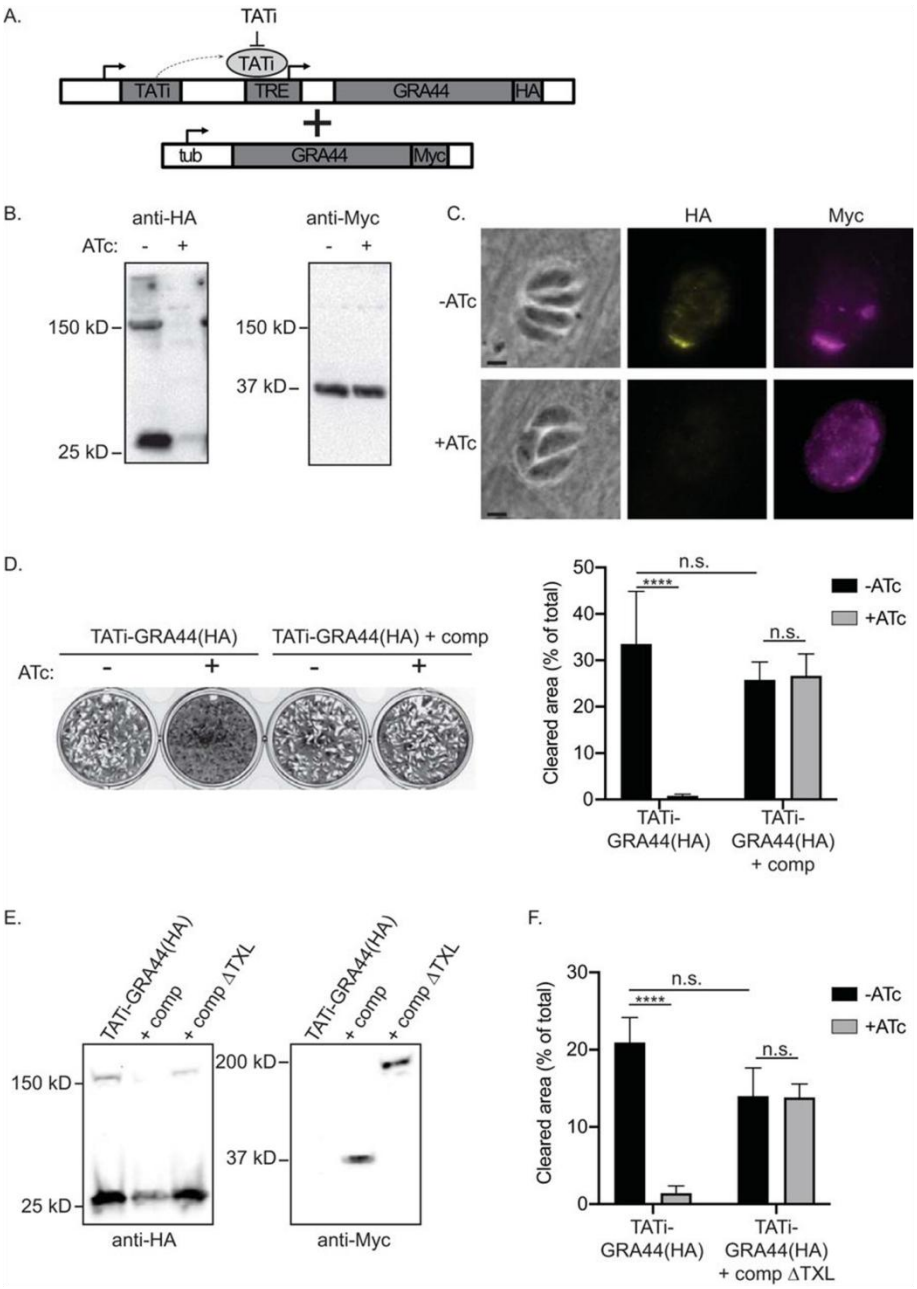


Figure 9. GRA44 knockdown complemented by rescue with WT GRA44.

To determine that the lack of GRA44 elicits the observed phenotype, a complemented line was established by adding an exogenous copy of wild-type GRA44 to the TATi-GRA44(HA) conditional knockdown line. (A) Schematic of strategy used for complementation. The wild-type copy of GRA44 added to the TATi-GRA44(HA) line contains a C-terminal MYC epitope tag and is driven by the *Toxoplasma* tubulin gene promoter (tub) in the TATi-GRA44(HA)comp parasite line. (B) Western blot of lysates from TATi-GRA44(HA)comp parasites grown with and without ATc for 48

hours. Blots were probed for HA and MYC. (C) Representative IFA images of TATi-GRA44(HA)comp with and without ATc treatment stained for the HA-tagged regulatable GRA44 and the MYC-tagged constitutively expressed exogenous copy. Bars=2 µm. (D) (Left) Plaque assays were performed with knockdown TATi-GRA44(HA) and the complemented TATi-GRA44(HA)comp parasites grown with or without ATc for 5 days. (Right) The average percentage of cleared cell monolayer from biological and experimental triplicates is shown on a bar graph (n=3; mean ± SD; ****, P<0.0001; n.s., not significant [significance was determined by one-way analysis of variance, followed by Tukey's test]). (E) TATi-GRA44(HA) was complemented with an exogenous copy of GRA44 containing a TEXEL deletion of amino acids 1348 to 1352 with a C-terminal MYC tag to give TATi-GRA44(HA)compΔTXL parasite line. Lysates from TATi-GRA44(HA), wild-type complemented strain TATi-GRA44(HA)comp, and ΔTXL complemented strain TATi-GRA44(HA)compΔTXL were analyzed by western blot and probed for HA and MYC. (F) Plaque assays were performed with the TATi-GRA44(HA) andTATi-GRA44(HA)compΔTXL lines. Parasites were grown 5 days with or without ATc. The average percentage of cell monolayer cleared for biological and experimental triplicates is shown on a bar graph (n=3; mean ± SD; ****, P<0.0001; n.s., not significant [significance was determined by one-way analysis of variance, followed by Tukey's test]).

Interaction with translocation machinery

Results presented thus far have shown GRA44 to be of significant importance for successful parasite propagation, but details about this protein's function and how it is important remain unclear. To better understand the context for GRA44 function, a comprehensive interactome of significant GRA44 protein interactors was established (Table 2). In short, GRA44 protein was isolated from GRA44(HA) parasite lysates by immunoprecipitation and peptides of co-precipitating proteins were analyzed by mass spectrometry. Experiments were done in triplicate for both anti-HA and nonspecific controls, and data statistically analyzed by significant analysis of interactome (SAINT) computational predictive

analysis. With a SAINT score of >0.8 used as a cutoff, a list of 35 putative interactors was generated. Of 35 interactors, 8 were ribosomal and snRNP proteins, which are likely of no importance to GRA44 function. Of the remaining 27 putative interactors, 23 had predicted signal peptides, indicating they are potentially secreted proteins. Among these were 8 known GRA proteins (GRA9, -16, -25, -33, -34, -45, -50,and -52); the PV membrane-associated protein MAF1; and MYR1, a member of the effector translocation system.

Protein	Product description
TGGT1_316250	GRA45
TGGT1_204340	GRA54
TGGT1_254470	MYR1
TGGT1_319340	GRA52
TGGT1_279100	MAF1a
TGGT1_251540	GRA9
TGGT1_203600	GRA50
TGGT1_304955	Serine/threonine-specific protein phosphatase (PPM11C)
TGGT1_315610	Hypothetical protein
TGGT1_203290	GRA34
TGGT1_270320	Protein phosphatase 2C domain-containing protein (PPM3C)
TGGT1_258870	Hypothetical protein
TGGT1_311720	Chaperonin protein BiP
TGGT1_226240	Putative bud site selection protein
TGGT1_216770	Hypothetical protein
TGGT1_220950	MAF1b
TGGT1_270240	MAG1
TGGT1_200360	Hypothetical protein
TGGT1_290700	GRA25
TGGT1_258458	Hypothetical protein
TGGT1_262050	Rhoptry kinase family protein ROP39
TGGT1_410360	MAF1 copy
TGGT1_247440	GRA33
TGGT1_229480	Putative calcium binding protein precursor
TGGT1_208830	GRA16
TGGT1_410370	MAF1 copy

Table 2. GRA44 interactome.
Top protein interactors of GRA44 in order of interaction significance as determined by SAINT.

To confirm the interaction with MYR1, for which there are antibodies, coimmunoprecipitation (co-IP) assays were performed (Figure 10). Purified lysate was precipitated fromGRA44(HA) parasites on mouse anti-HA beads and

controls done similarly with nonspecific mouse IgG beads. Eluates were evaluated by western blotting for presence of both GRA44 (Figure 10A) and MYR1 (Figure 10B and C). For MYR1, which is also processed by ASP5 at an internal TEXEL site, samples were probed with antibodies for either C-terminal or N-terminal cleavage products. Western analysis of eluate from GRA44 immunoprecipitation yielded significant amounts of both MYR1 segments compared to that in control eluate from the same source. This data would suggest that GRA44 interacts with MYR1, a protein with known importance to effector translocation.

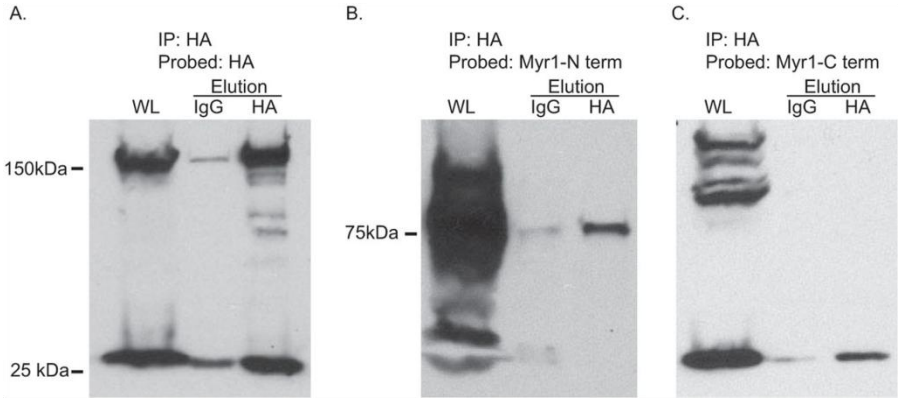


Figure 10. MYR1 immunoprecipitates with GRA44.
Beads conjugated to HA antibodies were used to IP GRA44(HA) with mouse IgG beads used as a nonspecific control. Eluate sample probed for HA by Western blot showed successful IP of GRA44 (A). Probing for MYR1 yielded bands for both N-terminal (B) and C-terminal (C) domains in the GRA44 eluate.

Host cMyc induction

Identification of MYR1 as a protein important to effector translocation was accomplished by a forward genetic screen of parasite mutants exhibiting translocation defects and consequential loss of host nuclear cMyc upregulation. Under normal conditions, wild-type parasites upregulate the host oncogenic

factor cMyc by translocation of GRA16 and GRA24 to the infected host cell's nucleus. Given GRA44's interaction with MYR1, it was plausible that it may be involved in similar functions related to translocation. To determine if this was indeed the case, TATi-GRA44(HA) parasites were assessed for their ability to induce host nuclear cMyc upregulation under knockdown conditions (Figure 11). To do this, parasites were first grown 24 hours with ATc, released from the host cells, and allowed to infect new cells under constant ATc conditions. After 12 hours of growth in the new host cells, cultures were fixed and an IFA for human cMyc was performed (Figure 11A). Control cultures were grown and treated identically with the exception of ATc, which was omitted from all steps. Images obtained by merged phase-contrast microscopy and HA, MYC, DAPI (4,6-diamidino-2-phenylindole), and cMyc staining were used to locate infected host cells with only those cells infected by single PVs with greater than 1 parasite per vacuole used for analysis. Single-channel images of cMyc staining within these host nuclei boundaries were quantitated with ImageJ software (Figure 11B). Addition of ATc to TATi-GRA44(HA) parasites significantly reduced GRA44 levels and reduced the cMyc signal by approximately 5-fold, a significantly subdued response compared to the control assay. No significant differences in the induced cMyc signal were observed in the complemented strain under ATc knockdown conditions. The GRA44 complement has also been successfully used to rescue GRA16 host-nuclear transolocation inhibition, as observed by IFA, which occurs in a GRA44 knockout line as described by Cygan et al[68].

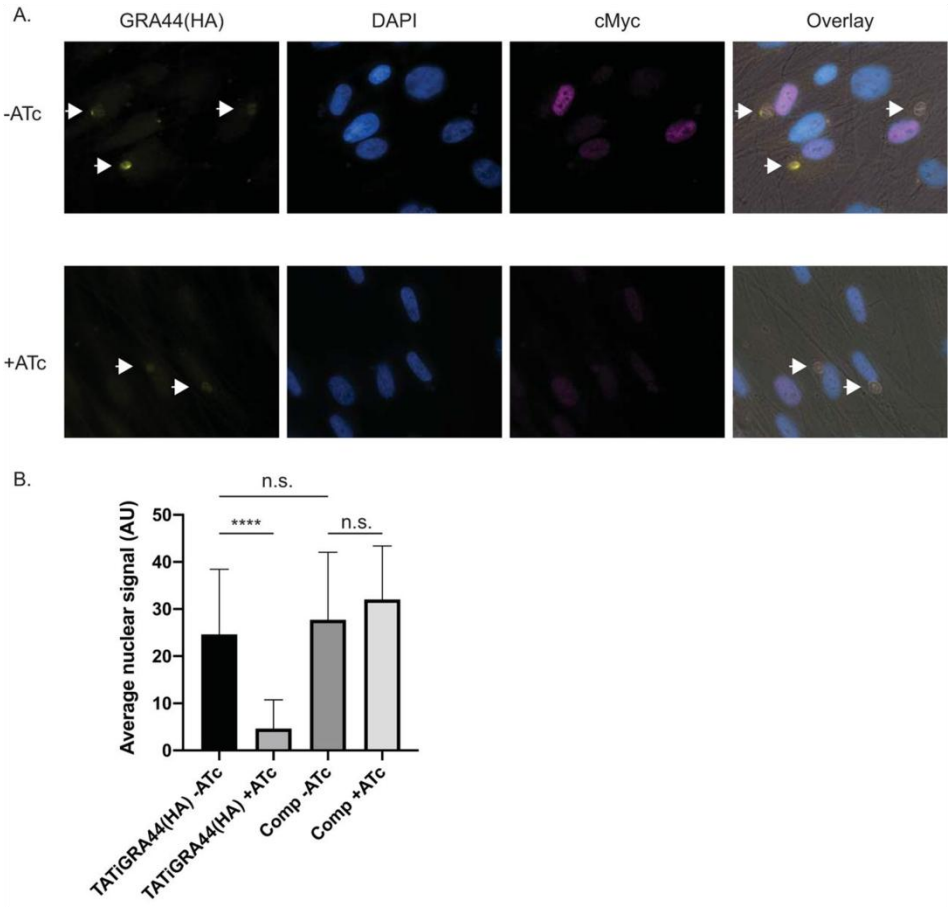


Figure 11. Host cMyc induction is dependent on GRA44.
Host nuclear cMyc expression was quantified by fluorescence microscopy after invasion by either the knockdown TATi-GRA44(HA) or the complemented knockdown TATi-GRA44(HA)comp parasite lines in the presence and the absence of ATc. (A) Representative IFA images of TATI-GRA44(HA) parasites grown without (- ATc) or with (+ ATc). Cultures were stained for HA to detect GRA44 (yellow), DAPI to detect nuclei (blue),and host cMyc (magenta). Arrows point at PVs containing more than 2 parasites. (B) Graph represents the average quantified nuclear signal from cMyc antibody staining across biological and experimental triplicates. Arbitrary units (AU) were used in comparing the nuclear cMyc signal intensity (n = 3; ****, P<0.0001; n.s., not significant [significance was determined by one-way analysis of variance, followed by Tukey's test]).

Measuring GRA44 phosphatase activity

The putative phosphatase domain of GRA44 was recombinantly expressed in *E. coli* with a C-terminal tag featuring a chitin binding domain (CBD)

and internal intein domain. The CBD tag facilitates protein purification by affinity column with a chitin resin and allows for quick elution via reduction-dependent intein cleavage. The presumed sequence of the GRA44 phosphatase domain was fused to the CBD tag and expressed in Rosetta cells (Figure 12). Two concentrations of IPTG were assessed for effectiveness, although none of the recombinant protein appeared soluble upon western blot analysis (Figure 12B). No significant difference in amounts or solubility of protein was observed using either 0.4 mM or 1.0 mM IPTG for induction.

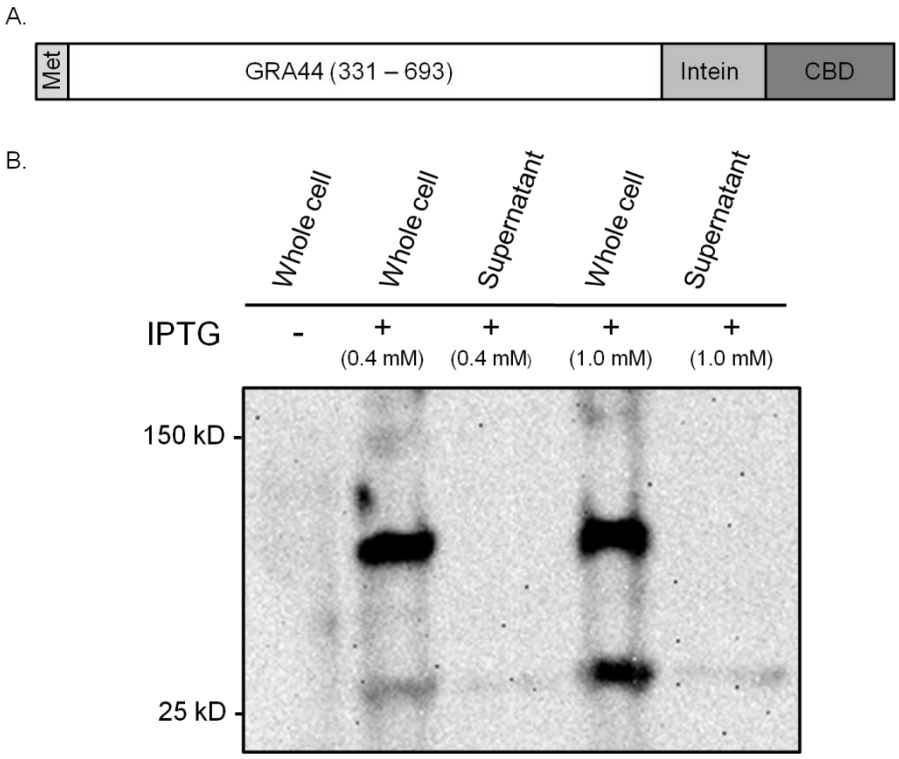


Figure 12. Recombinant GRA44 domain was expressed in *E. coli.* (A) Schematic of GRA44 expression construct containing putative phosphatase domain (amino acids 331 to 693) fused to intein-CBD tag.(B) Induction samples were separated by centrifugation to isolate soluble protein and analyzed by Western blot.

Due to difficulties in expressing recombinant GRA44 protein for the purpose of assaying phosphatase activity, an alternative approach was also

pursued. For this alternative approach, GRA44 was assayed from GRA44(HA) parasite lysate by a modified ELISA method (Figure 13). Rabbit anti-HA antibodies were affixed to a 96 well plate, blocked with nonspecific BSA protein, and incubated with parasite lysate. This was followed by washing and incubation with *para*-nitrophenyl phosphate (PNPP) substrate. The method was first tested with a commercial goat anti rabbit alkaline phosphatase antibody conjugateas a positive control. The goat anti rabbit antibody was successful in binding the rabbit anti HA antibodies of the ELISA plate and produced a significant amount of cleaved pNPP substrate at pH 8, as measured by absorbance at 405 nm (Figure 13A). ELISA plates were incubated with purified GRA44(HA) parasite lysate at different pH values (Figure 13B). Purified lysate from untagged parental parasites was included as a negative control in addition to alkaline phosphatase (AP) antibody conjugate for a positive control. Absorbance measurements yielded no significant differences between GRA44(HA) and parental samples, suggesting a lack of any enriched phosphatase activity in those samples. The positive control showed a large increase in A405 as expected.

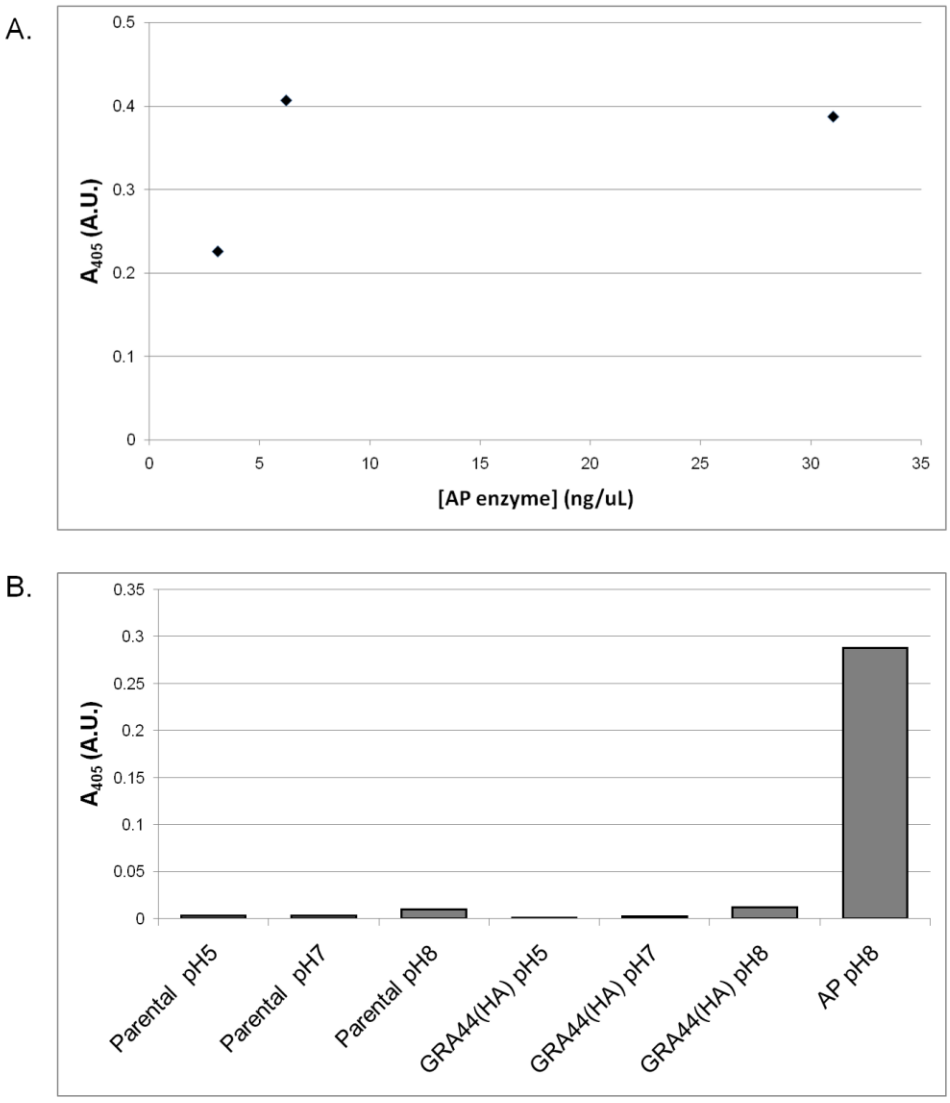


Figure 13. Phosphatase activity of parasite lysate.
(A) Positive control goat anti rabbit alkaline phosphatase conjugate showed increasing phosphatase activity with increasing enzyme concentration at pH = 8 (N=1). (B) Parental and GRA44(HA) lysates showed no activity at different pH, while the alkaline phosphatase positive control showed significant cleavage of pNPP substrate (N=1).

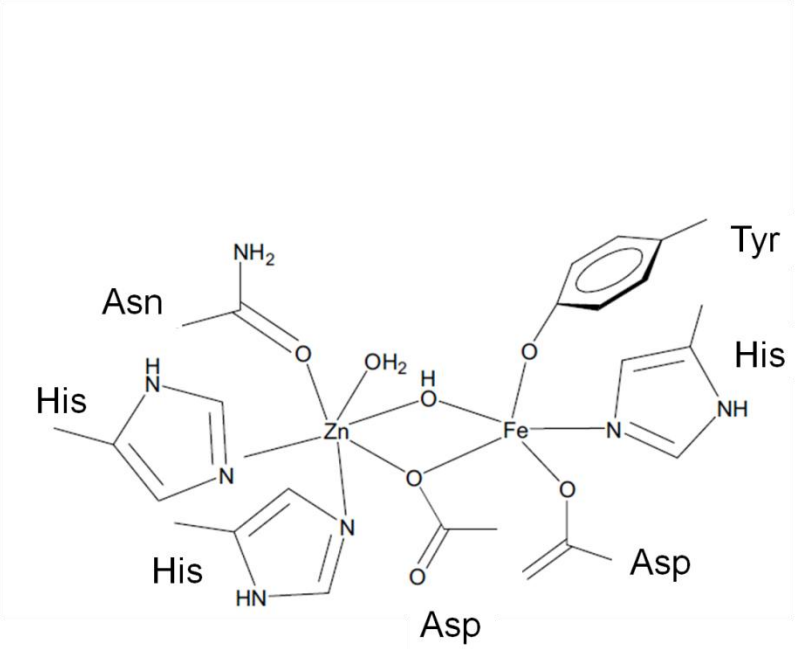
Full experimental datasets and uncropped figures are available as supplemental information to previously published work[55].

Chapter 4: Discussion

The presented data strongly suggest GRA44 to be a PV-resident protein with an essential cellular function likely correlated to *Toxoplasma's* global secretory pathway. The secretory pathway is required for translocation of parasite effector proteins to the host cell where they induce status changes to host processes. Parasite-driven host changes largely follow a general trend of increased metabolism and growth, with dysfunctional innate immune and apoptotic pathways. Conceptually, sustaining host cell growth pathways and elevated metabolism is analogous to an induced state of cellular overdrive, which helps provide an intracellular environment richer in essential biological molecules such as nulceotides, metabolic intermediates and simple lipids. Simultaneously suppressing host innate immune function and apoptosis allows *Toxoplasma* to replicate unhindered, while within an artificially enriched environment. An essential component of the translocation system is the MYR1/2/3 complex, however mechanistic details remain unknown. MYR1 and MYR3 are known to directly associate through presumed protein interactions, while MYR2 likely is a more transient interacting member[32]. Many of *Toxoplasma's* secreted proteins are highly disordered in nature, so an active transport system would be probable, although this is largely speculation.

Knockdown of GRA44 resulted in significant reduction of host cMyc activation, a similar phenomenon observed in MYR1 knockout parasites. This result combined with strong evidence of direct association with MYR1 supports the notion that GRA44 is a participating member of the MYR1/2/3 translocation

system and that GRA44 interaction with MYR1 is functionally relevant. Additional work by Cygan et al. further corroborates the idea of GRA44 being essential to parasite secretion by showing that GRA44 knockout parasites are unable to secrete the effector GRA16 to the host, a defect rescued by transient expression of wild-type GRA44. Whether GRA44 functions as a structural or regulatory protein remains unknown and requires further investigation. The possibility that GRA44 acts in a regulatory capacity is supported by the presence of a putative catalytic site reminiscent of acid phosphatases. In addition to multiple classes of phosphatases, *Toxoplasma* also expresses pseudophosphatases, catalytically inactive enzymes theorized to function through binding of certain key targets within cellular pathways. This compares to pseuodokinases, which have been observed to be secreted as a means to affect host systems. Whether GRA44 is an active phosphatase or a pseudophosphatase is unknown, but either type has potential to impact parasite propagation. In the biology of animals, plants, and fungi, acid phosphatases similar to GRA44 serve many biological purposes, and proteins are characterized by a shared catalytic site structural arrangement (Figure 14)[46,69]. These enzymes coordinate two metals in the active site, usually an Fe(III) and another metal such as Mn(II), Zn(II), or Fe(II). Each metal is separately coordinated to 3 amino acids with a shared aspartic acid bridge between them. The Fe(III) atom is typically coordinated to a histidine, aspartate, and tyrosine with the divalent metal atom coordinated by two histidines and an asparagine [35, 36].



Modified from Schenk et al., Crystal structures of a purple acid phosphatase representing different steps of this enzyme's catalytic cycle. BMC Structural Biology 2008, 8:6, doi:10.1186/1472-6807-8-6

Figure 14. Active site of acid phosphatase.
The active site of red kidney bean purple acid phosphatase is represented to illustrate the coordination of metal atoms with seven key amino acids.

From phosphatase domain homology alignments, GRA44 appears to contain a majority of the conserved residues common to acid phosphatases, but the aspartate conjugating the Fe(III) is replaced by an asparagine and the asparagine conjugating the second metal is replaced by a glutamate (Figure 15)[69]. This would represent a swap in charged amino acids, but still maintains a stable charge equilibrium within the active site. The only coordinating residue unaccounted for is the tyrosine binding Fe(III). The active site of GRA44 is then hypothesized to consist of the first metal, M(II), coordinated by glutamate and two histidines, and the second metal, M(III), bound by an asparagine, histidine, and unknown seventh residue with both metals sharing an aspartate bridge.

Figure 15. GRA44 has residues common to acid phosphatases.
In the GRA44 phosphatase domain (GRA44 PD), there is an apparent swap of the N and E amino acids between metal coordinating sites. Residues coordinating the first metal are marked (#) and second metal (*). The shared aspartate bridge is annotated by (=).

The cellular roles of acid phosphatases vary greatly, but are usually centered around scavenging, recycling, and transport of inorganic phosphorus. In humans, acid phosphatases have been ascribed function in cell signaling, such as downregulation of prostate cell growth and osteoclast bone resorption activity[45,46]. In plants, acid phosphatases have been implicated in phosphate acquisition from organophosphate compounds and protein dephosphorylation of enzymes[47,48,50]. In *Toxoplasma*, GRA44 function is likely related to its interactions

with the MYR translocon in the PV, where it could regulate MYR proteins by protein dephosphorylation activity. The MYR3 protein has been observed in a phosphorylated form, indicating a dynamic phosphorylative status[31]. ROP17, a secreted kinase, has been shown critical for efficient effector translocation, which is consistent with the idea of phosphoregulation of the translocation system[70]. GRA44 could plausibly have a role in this hypothetical phosphoregulation, either through MYR3 or other proteins. Alternatively, GRA44 could be important for dephosphorylating effectors prior to trafficking across the PVM barrier. For transport across a lipid bilayer membrane, such as the PVM, proteins may require dephosphorylation. Notably, both GRA16 and GRA24 have been shown to exist primarily as phosphorylated proteins, as identified by a phosphoproteome analysis in Toxoplasma[71].

Although GRA44 knockdown results in defective host cMyc activation and a significant propagation defect, disruption of other translocon members does not adversely affect propagation to a similar degree. The relative fitness scores assigned to these genes by Sidik et al reflect this difference, where the score for GRA44 is -3.28 the relative fitness scores for MYR1, MYR2, and MYR3 are 0.88, 2.39, and 2.83, respectively[67]. This discrepancy is peculiar because disruption of MYR1, MYR2 or MYR3 interferes with effector translocation and cMyc activation. Additionally, the MYR1-dependent effectors GRA16, GRA24, and TgIST also have positive knockout fitness scores of 1.44, 2.28, and 2.86, respectively. Consequently, it must be concluded that the propagation defect exhibited by parasites lacking GRA44 is unlikely caused by defects to effector translocation.

An alternative explanation to GRA44's importance regarding propagation and its link to translocation is that GRA44 has multiple independent roles, one of which is nutrient acquisition like many other common smaller-sized acid phosphatases[72]. A multipurpose functionality would be consistent with the compiled results of this study and others.

Chapter 5: Conclusion and future directions

Conclusions

During its life cycle, *Toxoplasma* invades and replicates within host cells, where nutrient resources are readily available and consistently produced. To better facilitate their life cycle, *Toxoplasma* employs many means of altering the status of their host to better suit their needs. Examples of parasite-driven host alteration include apoptosis inhibition, innate immune system disruption, host cytoskeleton restructuring, and global changes to host gene transcription[26,28,32,73–75]. Host cell alterations such as these are accomplished by a specialized arsenal of parasite effector proteins secreted into the host cell during all stages of parasite development. Most of these effectors are secreted by one of two unique parasite organelles: the rhoptries and the dense granules. Rhoptries discharge the majority of proteins known as ROPs and RONs during invasion, but some ROPs are secreted during parasite replication. Dense granules release their contents into the PV after invasion, during intracellular division and parasite growth. While some of these secreted proteins remain within the PV lumen or associate with the PVM, many are translocated across the PVM into the host, where they affect numerous host functions. The mechanism of protein secretion from PV to host has become clearer with the discovery of associated proteins appearing to comprise components of a translocation system[30,31]. My work on the protein GRA44 (TGGT1_228170), previously named as IMC2A[54], has identified it as an additional interacting member of this system, whose presence is critical for host alteration events

downstream of effector translocation. This study showed GRA44 to be necessary for host cMyc activation, one of many alterations imposed on the host by *Toxoplasma*. GRA44 was also found to be essential for parasite propagation, a characteristic not seen in the other translocon system members. In addition, this study showed GRA44 to be cleaved at an internal TEXEL site located at residues 1348 to 1350 and secreted into the PV. Interruption of cleavage at this site had no inhibitory effects to GRA44 secretion or parasite propagation. Interestingly, both major GRA44 cleavage products were observed to be secreted into the PV where they largely colocalized. This is important, as the fragment containing the putative acid phosphatase is part of the N-terminal cleavage product, which was seen localizing in the PV lumen and PVM. The fact that TEXEL processing was not necessary for either the secretion or function of GRA44 is consistent with reports of other secreted proteins containing TEXEL motifs, such as MYR1 and WNG1/2, which can still be secreted even in the absence of ASP5 protease activity[27]. By chance, the GRA44 knockdown line presented with a spontaneous gene deletion likely occurring from a local DNA recombination event, however this would prove an unexpectedly fruitful situation since it made clear that the last 105 amino acids of the protein are not needed for function or localization. This finding suggests the C-terminus of GRA44 may not confer any function, a concept in line with the fact that C-terminal TEXEL cleavage has no effects to protein functionality. Analysis of proteins co-immunoprecipitating with GRA44 revealed interactions with many known secreted proteins, including numerous GRAs. Among these interactors were GRA9, part of the intravacuolar tubular

network; GRA16, an effector altering the host cell cycle through the p53 pathway; GRA25, a macrophage dependent immune modulator, and members of the multicopy mitochondrion association factor 1 (MAF1) family of proteins essential for recruiting host mitochondria to the PVM surface[29,76–78]. The proteins GRA33, GRA34, GRA45, GRA50, GRA52 which have yet to be functionally characterized were also found to interact with GRA44. Most importantly, immunoprecipitation revealed an interaction between MYR1 and GRA44, which was independently confirmed by western blotting (Figure 10). While studying the interactome of GRA44, I learned of ongoing work in John C. Boothroyd's lab showing a physical and functional interaction between MYR1 and GRA44[68]. MYR1 was initially identified through a forward genetic screen for Toxoplasma mutants unable to activate host c-Myc and translocate the effectors GRA16 and GRA24[30]. Further screening for such mutants identified MYR2 and MYR3[31]. Together MYR1/2/3 appear to be part of a putative translocon complex, although only MYR1 and MYR3 have been confirmed to directly interact by western blot. MYR1-dependent effectors are responsible for driving a broad range of host cell alterations including the upregulation of E2F transcription factors and the downregulation of interferon signaling[32].

In conclusion, I have shown GRA44 to be a secreted protein, critical for *Toxoplasma* propagation and host manipulation, that is an additional member to the MYR1/2/3 tranclocation complex through direct protein interactions with the translocon protein MYR1. Prior to secretion, GRA44 is cleaved at an internal TEXEL site, forming two stable and colocalizing proteins. The mechanistic action

by which GRA44 functions in regards to protein secretion to host cells remains unknown, however, due to its putative acid phosphatase domain, involvement with the dephosphorylation of trafficked proteins or members of the translocon complex is a plausible explanation.

Future directions

Further study of GRA44 and its role in parasite propagation will necessitate characterization of any enzymatic properties of the protein. Expression of the phosphatase domain in a eukaryotic system such as insect cells or yeast could expedite this inquiry, although the fact that GRA44 is a metal-dependent protein specific to *Toxoplasma* likely requiring specific chaperonins for correct conformational folding remains an obstacle. For future work, complementation of GRA44 knockdown parasites with exogenous GRA44 containing point mutations of key active site residues would allow for study of certain amino acids and their importance to function. Additionally, further development of the parasite lysate ELISA method for phosphatase activity measurements could yield more detailed enzymatic information of GRA44. In this work, all experiments have been conducted in Type I *Toxoplasma*, a strain unable to assume the bradyzoite stage and efficiently form cysts. Type II parasites are able to fully change stages between tachyzoite and bradyzoite, and therefore express a multitude of bradyzoite-specific proteins. Study of GRA44 in type II parasites may uncover more specific phenotypic results, since this parasite strain exhibits a more dynamic transcriptional profile involving gene sets exclusive to the bradyzoite life stage. Future work on enzymatic activity of

GRA44, potential substrates, and phenotypic data from other parasite stages will aid in uncovering more information on the regulation of effector translocation in *Toxoplasma*, a fundamental process to the interactions between parasites and their host.

Chapter 6: References

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Curriculum vitae

William James Blakely

Education

DOCTOR OF PHILOSOPHY

Indiana University, IUPUI (Indianapolis, IN)

November 2020

Major: Biochemistry and Molecular Biology

Thesis: Characterization of a Putative Phosphatase in Toxoplasma gondii and its Role in Parasite Propagation

Mentor: Gustavo Arrizabalaga

BACHELOR OF SCIENCE

Kutztown University of Pennsylvania (Kutztown, PA)

May 2013

Major: Chemistry and Biochemistry

Cumulative GPA: 3.55/4.00

Research Experience

GRADUATE STUDENT

Department of Biochemistry and Molecular Biology

Department of Pharmacology and Toxicology

Indiana University School of Medicine (Indianapolis, IN)

May 2016 - September 2020

Mentor: Gustavo Arrizabalaga, Ph.D.

Area of study: Secretory pathway and host interactions of the obligate intracellular parasite Toxoplasma gondii

UNDERGRADUATE RESEARCHER

Department of Chemistry

Kutztown University (Kutztown, PA)

January 2010 - June 2013

Mentors: Matthew Junker, Ph.D. and Carsten Sanders, Ph.D.

Area of study: Structure and activity of human cytochrome c synthetase

SUMMER UNDERGRADUATE RESEARCHER

Department of Chemistry

University of California Irvine (Irvine, CA)

July 2012 - September 2012

Mentor: Matthew Law, Ph.D.

Area of Study: Oxide Nanocrystal synthesis for dye-sensitized photocathode of a water-splitting fuel cell

Professional experience

QUALITY ASSURANCE INSPECTOR

Sharp Packaging Solutions, Inc (Conshohocken, PA)

September 2013 - July 2015

- Quality assurance oversight of pharmaceutical packaging operations
- Collaboration with Production personnel and Validation engineers
- Ensured compliance with FDA, DEA, EMEA regulations and cGMP standards
- Reviewed and verified product record and operations documentation

Teaching experience

RESEARCH MENTOR

Arrizabalaga Laboratory, Indiana University School of Medicine (Indianapolis, IN)

May 2018 – March 2020

Mentored two undergraduate biology students on projects related to a kinase-mediated signaling pathway of the Toxoplasma gondii cell cycle

UNDERGRADUATE TUTOR

Bucks County Community College (Newtown, PA)

May 2009 – June 2010

Provided one-on-one and group tutoring for students in basic, general, and organic chemistry courses

Publications

- Blakely W. J., Holmes M. J., Arrizabalaga G. (2020). The secreted acid phosphatase domain-containing GRA44 from Toxoplasma gondii is required for C-myc induction in infected cells. mSphere, 5 (1) e00877-19. doi: 10.1128/mSphere.00877-19
- Yang, C., Broncel, M., Dominicus, C., Sampson, E., Blakely, W. J., Treeck, M., &Arrizabalaga, G. (2019). A plasma membrane localized protein phosphatase in Toxoplasma gondii, PPM5C, regulates attachment to host cells. Scientific reports, 9(1), 5924.doi:10.1038/s41598-019-42441-1

Presentations

- Blakely, Holmes, Arrizabalaga (Sept. 2018) Post-translational Processing of a Secreted Putative Phosphatase in Toxoplasma gondii. Molecular Parasitology Meeting at Marine Biological Laboratory, Woods Hole MA
- Blakely, Holmes, Arrizabalaga(Sept.2017)Investigation of IMC2A: A Secreted Phosphatase of Toxoplasma gondii. Molecular Parasitology Meeting at Marine Biological Laboratory, Woods Hole MA
- Blakely, Kostomiris, Junker, Sanders(Apr. 2013) The Molecular Mechanism of HemeAttachment by Cytochrome c HemeLyase. 24th Annual Saint Joseph's University Sigma Xi Student Research Symposium, Philadelphia PA
- Fredericks, Weller, Hoppes, Blakely, Junker, Sanders(2012) Developing an in vitro system for determining the biochemical mechanism of apoptosis regulation by human holocytochrome c synthetase. Pennsylvania Academy of Science (PAS) 88th Annual Meeting, Allentown PA

Awards

Recipient of Carole and Ray Neag Undergraduate Research Grant in May 2012